

**HUMAN UROTHELIAL CELL SPECIFIC UROPLAKIN
TRANSCRIPTIONAL REGULATORY SEQUENCES, VECTORS
COMPRISING UROPLAKIN-SPECIFIC TRANSCRIPTIONAL
REGULATORY SEQUENCES, AND METHODS OF USE THEREOF**

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TECHNICAL FIELD

The invention provides new human DNA sequences which confer urothelial-cell specific expression on heterologous genes. Additionally, the invention relates to cell transduction using adenoviral vectors, and more particularly to adenoviral vectors which replicate preferentially in urothelial cells.

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BACKGROUND

Approximately 51,200 new cases of bladder cancer are diagnosed each year in the United States. Of these, approximately 38,000 cases are in men and 13,200 in women. Of the 51,200 new cases, approximately 80 percent will be classified at diagnosis as superficial, i.e., the cell have not invaded the muscularis propria. Of these, approximately 10 to 15 percent will eventually progress to invasive disease. The estimated number of deaths from bladder cancer in the United States in 1994 was 7,000 in men and 3,600 in women.

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In the United States, transitional cell carcinoma (TCC) accounts for 90 to 95 percent of all tumors of the bladder. Squamous cell carcinoma (SCC) represents 5 to 10 percent, and adenocarcinoma approximately 1 to 2 percent. Squamous cell and adenomatous elements are often found in association with transitional cell tumors, especially with high grade tumors.

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Bladder cancer is generally divided into superficial and invasive disease. A critical factor is the distinction between those tumors that are confined to the mucosa and those that have penetrated the basement membrane and extended into the lamina propria. The term "superficial bladder tumor" is generally used to represent a tumor that has not invaded the muscularis. Invasive tumors are described as those that have

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invaded the muscularis propria, the perivesical fibroadipose tissue, or adjacent structures. Carcinoma in situ (CIS) is a high grade and aggressive manifestation of TCC of the bladder that has a highly variable course.

Management of bladder cancer depends on whether the cancer is superficial or invasive. Most patients with superficial bladder cancer can be adequately treated with transurethral resection or fulguration of the tumor. However, with superficial bladder cancer, recurrence is the rule, and about 71 percent of patients develop tumor recurrences after endoscopic resection, and of these, about half experience recurrence within one year after the original resection. Agents used to treat CIS include bacille Calmette-Guérin (BCG) and chemotherapeutic agents, including doxorubicin. Response rates of up to 70 percent have been reported with BCG. However, treatment is not successful in all CIS patients, and some have progression to invasive or metastatic disease. Treatment of muscle-invasive bladder cancer includes radical cystectomy. Combination chemotherapy is generally used to treat metastatic bladder cancer, but success rates are dismal. Approximately 50 percent of patients with high-grade bladder cancer and deep muscle invasion die of disseminated disease within two years of presentation.

A number of urothelial cell-specific proteins have been described, among which are the uroplakins. Uroplakins (UP), including UPIa and UPIb (27 and 28 kDa, respectively), UPII (15 kDa), and UPIII (47 kDa), are members of a group of integral membrane proteins that are major proteins of urothelial plaques. These plaques cover a large portion of the apical surface of mammalian urothelium and may play a role as a permeability barrier and/or as a physical stabilizer of the urothelial apical surface. Wu et al. (1994) *J. Biol. Chem.* 269:13716-13724. UPs are bladder-specific proteins, and are expressed on a significant proportion of urothelial-derived tumors, including about 88% of transitional cell carcinomas. Moll et al. (1995) *Am. J. Pathol.* 147:1383-1397; and Wu et al. (1998) *Cancer Res.* 58:1291-1297. The control of the expression of the human UPII has been studied, and a 3.6-kb region

upstream of the mouse UPII gene has been identified which can confer urothelial-specific transcription on heterologous genes (Lin et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:679-683). See also, U.S. Patent Nos. 5,824,543 and 6,001,646.

Of particular interest is development of more specific, targeted forms of cancer therapy, especially in cancers that are difficult to treat successfully, such as bladder cancer. In contrast to conventional cancer therapies, which result in relatively non-specific and often serious toxicity, more specific treatment modalities attempt to inhibit or kill malignant cells selectively while leaving healthy cells intact.

One possible treatment approach for cancers such as bladder cancer is gene therapy, whereby a gene of interest is introduced into the malignant cell. A variety of viral and non-viral (e.g., liposomes) vehicles, or vectors, have been developed to transfer these genes. Of the viruses, retroviruses, herpes simplex virus, adeno-associated virus, Sindbis virus, poxvirus, and adenoviruses have been proposed for gene transfer with retrovirus vectors or adenovirus vectors being the focus of much current research. Adenoviruses are among the most easily produced and purified, whereas retroviruses are unstable, difficult to produce and to purify, and may integrate into the host genome, raising the possibility of dangerous mutations. Moreover, adenovirus has the advantage of effecting high efficiency of transduction and does not require cell proliferation for efficient transduction of cell. For general background references regarding adenovirus and development of adenoviral vector systems, see Graham et al. (1973) *Virology* 52:456-467; Takiff et al. (1981) *Lancet* 11:832-834; Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Graham (1984) *EMBO J* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; and Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

When used as gene transfer vehicles, adenovirus vectors are often designed to be replication-defective and are thus deliberately engineered to fail to replicate in the target cells of interest. In these vehicles, the early adenovirus gene products E1A and/or E1B are deleted and provided *in trans* by the packaging cell line 293. Graham

et al. (1987) *J. Gen. Virol* 36:59-72; Graham (1977) *J. Genetic Virology* 68:937-940. The gene to be transduced is commonly inserted into adenovirus in the E1A and E1B region of the virus genome. Bett et al. (1994). Replication-defective adenovirus vectors as vehicles for efficient transduction of genes have been described by, inter alia, Stratford-Perricaudet (1990) *Human Gene Therapy* 1:241-256; Rosenfeld (1991) *Science* 252:431-434; Wang et al. (1991) *Adv. Exp. Med. Biol.* 309:61-66; Jaffe et al. (1992) *Nat. Gen.* 1:372-378; Quantin et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584; Rosenfeld et al. (1992) *Cell* 68:143-155; Stratford-Perricaudet et al. (1992) *J. Clin. Invest.* 90:626-630; Le Gal Le Salle et al. (1993) *Science* 259:988-990; Mastrangeli et al. (1993) *J. Clin. Invest.* 91:225-234; Ragot et al. (1993) *Nature* 361:647-650; Hayaski et al. (1994) *J. Biol. Chem.* 269:23872-23875; Bett et al. (1994). Adenovirus E1A and E1B genes are disclosed in Rao et al. (1992, *Proc. Natl. Acad. Sci. USA* vol. 89: 7742-7746).

Until recently, the virtually exclusive focus in development of adenoviral vectors for gene therapy has been use of adenovirus merely as a vehicle for introducing the gene of interest, not as an effector in itself. Replication of adenovirus had previously been viewed as an undesirable result, largely due to the host immune response. More recently, however, the use of adenovirus vectors as effectors has been described. International Patent Application Nos. PCT/US98/04084, PCT/US98/04133, PCT/US98/04132, PCT/US98/16312, PCT/US95/00845, PCT/US96/10838, PCT/EP98/07380, U.S. Pat. No. 5,998,205, and U.S. Patent 5,698,443.

In the treatment of cancer by replication-defective adenoviruses, the host immune response limits the duration of repeat doses at two levels. First, the capsid proteins of the adenovirus delivery vehicle itself are immunogenic. Second, viral late genes are frequently expressed in transduced cells, eliciting cellular immunity. Thus, the ability to repeatedly administer cytokines, tumor suppressor genes, ribozymes, suicide genes, or genes which convert prodrug to an active drug has been limited by

the immunogenicity of both the gene transfer vehicle and the viral gene products of the transfer vehicle as well as the transient nature of gene expression.

There is a need for vector constructs that are capable of eliminating essentially all cancerous cells in a minimum number of administrations before specific immunological response against the vector prevents further treatment.

All publications cited herein are hereby incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present invention provides urothelial cell specific transcriptional regulatory sequences which regulate expression of the human uroplakin II (*hUPII*) gene, which can form part of an *hUPII* transcriptional regulatory element (*hUPII*-TRE). An *hUPII*-TRE in turn can be operably linked to a heterologous polynucleotide to effect transcriptional control of the linked gene.

The present invention provides vectors comprising an *hUPII*-TRE operably linked to a heterologous polypeptide which can be used to effect transcriptional control of the linked polypeptide in cells that respond to the *hUPII*-TRE. In particular, replication-competent adenoviral vectors specific for urothelial cells and methods for their use are also provided. In these replication-competent adenovirus vectors, one or more genes, preferably adenoviral genes, is under transcriptional control of a urothelial cell-specific transcriptional response element (TRE). Preferably, the adenoviral gene under transcriptional control of a urothelial cell-specific TRE is one that is essential for adenoviral propagation. A transgene under control of the urothelial cell-specific TRE may also be present.

Accordingly, the invention provides an isolated polynucleotide comprising 200 contiguous nucleotides of nucleotides 1 to 2239 of SEQ ID NO:1, preferably comprising bases about 2023 to about 2239 of SEQ ID NO:1 (but not depicted in SEQ ID NO:2), and having urothelial cell-specific TRE activity. The present

invention also provides an isolated polynucleotide comprising 200 contiguous nucleotides of nucleotides 430 to 2239 of SEQ ID NO:1, preferably comprising bases about 2023 to about 2239 of SEQ ID NO:1 (but not depicted in SEQ ID NO:2), and having urothelial cell-specific TRE activity. In another aspect, the invention provides an isolated polynucleotide comprising 200 contiguous nucleotides having at least about 70%, more preferably at least about 75%, 80%, 85%, 90%, 95%, 98% or 99%, sequence identity to a sequence within nucleotides 1 to 2239 of SEQ ID NO:2 (but not depicted in SEQ ID NO:1), with the polynucleotide having urothelial cell-specific TRE activity. In another aspect, the invention provides an isolated polynucleotide comprising a region of at least 20 contiguous nucleotides, with the region able to hybridize under stringent conditions to a polynucleotide comprising nucleotides about 1 to about 2239, about 430 to about 2239, about 2038 to about 2239, and/or about 1647 to about 2239 of SEQ ID NO:1, with the polynucleotide having urothelial cell-specific TRE activity (and not depicted in SEQ ID NO:2).

In another aspect, the invention provides an isolated polynucleotide comprising at least about 20 nucleotides which hybridize under stringent conditions to a region of SEQ ID NO:1, wherein the region is nucleotides about 1 to about 2239 of SEQ ID NO:1 or a complementary sequence thereof, wherein the at least about 20 nucleotides are not depicted in SEQ ID NO:2. In another aspect, the invention provides an isolated polynucleotide comprising at least about 20 nucleotides which hybridize under stringent conditions to a region of SEQ ID NO:1, wherein the region is nucleotides about 430 to about 2239 of SEQ ID NO:1 or a complementary sequence thereof, wherein the at least about 20 nucleotides are not depicted in SEQ ID NO:2. In various embodiments, the regions are nucleotides about 2028 to about 2239, about 430 to about 2239, about 1647 to about 2239, or about 1223 to about 2239 of SEQ ID NO:1.

In another aspect, the invention provides isolated polynucleotides of at least 20 contiguous nucleotides of SEQ ID NO:1.

In another aspect, the invention provides isolated polynucleotides comprising a transcriptional regulatory element which comprises a *hUPII* 5'-flanking polynucleotide sequence which confers urothelial cell-specific transcription on heterologous polynucleotide sequences.

5 The invention also provides vectors and/or delivery vehicles containing these *hUPII* urothelial cell-specific TRE polynucleotide(s). Such vectors and/or delivery vehicles can be introduced into cells both *in vivo* and *in vitro*.

10 In another aspect, the invention provides adenovirus vectors comprising co-transcribed first and second genes under control of a urothelial cell-specific TRE, wherein the second gene is under translational control of an internal ribosome entry site (IRES).

15 The invention also provides methods for introducing into a cell a vector and/or a delivery vehicle containing *hUPII* urothelial cell-specific TRE polynucleotide(s). The invention further provides host cells containing *hUPII* urothelial cell-specific TRE polynucleotide(s).

20 In other aspects, the invention provides methods of creating constructs comprising *hUPII* urothelial cell-specific TRE polynucleotide(s) operably linked to a heterologous polynucleotide and further provides methods for increasing the transcription and/or expression of the linked heterologous polynucleotide generally involving introducing the constructs into suitable cells.

25 Accordingly, the invention provides methods for increasing transcription of polynucleotide sequence in a cell comprising introducing a construct comprising *hUPII* urothelial cell-specific TRE polynucleotide(s) operably linked to said polynucleotide into a cell in which said *hUPII* urothelial cell-specific TRE polynucleotide(s) is functional.

 In another aspect, the invention provides an adenovirus vector comprising an adenovirus gene under transcriptional control of a urothelial cell-specific TRE. In another embodiment, a urothelial cell-specific TRE is human. In another

embodiment, a urothelial cell-specific TRE comprises a urothelial cell-specific promoter and a heterologous enhancer. In other embodiments, a urothelial cell-specific TRE comprises a urothelial cell-specific promoter. In other embodiments, a urothelial cell-specific TRE comprises a urothelial cell-specific enhancer and a heterologous promoter. In other embodiments, a urothelial cell-specific TRE comprises a urothelial cell-specific promoter and a urothelial cell-specific enhancer.

In some embodiments, the adenovirus gene under transcriptional control of a urothelial cell-specific TRE is an adenovirus gene essential for replication. In some embodiments, the adenoviral gene essential for replication is an early gene. In another embodiment, the early gene is E1A. In another embodiment, the early gene is E1B. In yet another embodiment, both E1A and E1B are under transcriptional control of a urothelial cell-specific TRE. In other embodiments, the adenovirus gene essential for replication is a late gene.

In some embodiments, the urothelial cell-specific TRE is derived from the 5' flanking region of a uroplakin gene. In some of these embodiments, the urothelial cell-specific TRE is derived from the 5' flanking region of a UPIa gene. In other embodiments, the urothelial cell-specific TRE is derived from the 5'-flanking region of a UPIb gene. In yet other embodiments, the urothelial cell-specific TRE is derived from the 5'-flanking region of a UPII gene. In yet other embodiments, the urothelial cell-specific TRE is derived from the 5'-flanking region of a UPIII gene.

In other embodiments, the invention provides an adenovirus vector comprising (a) an adenovirus gene under transcriptional control of a urothelial cell-specific TRE; and (b) an E3 region. In some of these embodiments the E3 region is under transcriptional control of a urothelial cell-specific TRE.

In another aspect, the invention provides a host cell comprising the adenovirus vector(s) described herein.

In another aspect, the invention provides pharmaceutical compositions comprising an adenovirus vector(s) described herein.

In another aspect, the invention provides kits which contain an adenoviral vector(s) described herein.

5 In another aspect, methods are provided for conferring selective cytotoxicity in target cells (i.e., cells which permit or induce a urothelial cell-specific TRE to function), comprising contacting the cells with an adenovirus vector(s) described herein, whereby the vector enters the cell.

10 In another aspect, methods are provided for propagating an adenovirus specific for urothelial cells, said method comprising combining an adenovirus vector(s) described herein with urothelial cells, whereby said adenovirus is propagated.

The invention further provides methods of suppressing bladder cancer cell growth, comprising contacting a bladder cancer cell with an adenoviral vector of the invention such that the adenoviral vector enters the bladder cancer cell and exhibits selective cytotoxicity for the bladder cancer cell.

15 In another aspect, methods are provided for detecting bladder cancer cells in a biological sample, comprising contacting cells of a biological sample with an adenovirus vector(s) described herein, and detecting replication of the adenovirus vector, if any.

BRIEF DESCRIPTION OF THE DRAWINGS

20 FIGS. 1A-1C depicts a nucleotide sequence of a human uroplakin II 5' flanking region. Position +1 (the translational start site) is denoted with an asterisk (in Fig. 1A, nucleotide number 1 represents nucleotide -2239; nucleotide number 430 represents nucleotide -1809).

25 FIGS. 2A-2E depicts a nucleotide sequence of a mouse uroplakin II 5' flanking region. The translational start site is denoted with an asterisk (in Fig. 2A, nucleotide number 1 represents nucleotide -3592).

FIGS. 3A-3B are a series of schematic depictions of various plasmid constructs described herein.

FIGS. 4A-4G are a series of schematic depictions of various adenoviral constructs described herein.

FIG. 5 is a bar graph depicting the results of an assay to assess the bladder specificity of CP648 (TRE for human uroplakin 1A with 2.0 kb flanking sequence 5' to luciferase reporter), CP649 (TRE for human uroplakin 1A with 1.0 kb flanking sequence 5' to luciferase reporter), CP662 (TRE for hUPII with 200 bp flanking sequence 5' to luciferase reporter), CP663 (TRE for human uroplakin II with 600 bp flanking sequence 5' to luciferase reporter), CP656 (TRE for human uroplakin II with 1 kb flanking sequence 5' to luciferase reporter), CP657 (TRE for human uroplakin II with 2.3 kb flanking sequence 5' to luciferase reporter), and CP620 (TRE for mouse uroplakin II with flanking sequence -3531 to +60 that is 5' to luciferase reporter) under the control of human uroplakin II promoter in different cell lines.

FIG. 6 is a bar graph depicting results of a luciferase assay to indicate bladder specificity of plasmids CP618 (0.6 kb mUPII, first bar); CP619 (1.0 kb mUPII, second bar); CP1010 (2 kb mUPII, third bar); CP656 (1 kb hUPII, fourth bar); CP657 (2.2 kb hUPII, fifth bar); and CP620 (3.6 mUPII, six bar) in various cell lines.

FIG. 7 shows a schematic representation of the E3 region of adenovirus.

FIG. 8 is a bar graph depicting the results of a viral replication assay for viral constructs CV702 (E3-deleted adenovirus; hatched bar) and CV808 (E3-deleted adenovirus with E1A and E1B with endogenous promoter under the control of a mUPII ; solid bar) in various cell lines. The star over PA-1 indicates no replication was observed.

FIG. 9 is a bar graph depicting the results of a viral replication assay for viral constructs CV802 (first bar) and CV829 (second bar).

FIG. 10 is a bar graph depicting the results of a viral replication assay for viral construct CV829 in SW780 and smooth muscle cells.

FIG. 11 is a graph depicting change in tumor volume of bladder xenografts in nude mice treated with CV808 (solid boxes) versus vehicle alone (no virus; solid diamonds).

FIG. 12 provides a nucleic acid and amino acid sequence for ADP.

5 FIG. 13 depicts an E3 region.

FIG. 14 shows the virus yield of CV802, CV882 and CV884 in cell lines.

MODES FOR CARRYING OUT THE INVENTION

10 We have isolated and characterized a 2.2 kb 5'-flanking DNA sequence of the human uroplakin gene and a 1.8 kb 5'-flanking DNA sequence of the human uroplakin gene both of which regulate, in a tissue-specific manner, transcription of human uroplakin II (*hUPII*; Fig. 1 (SEQ ID NO:1)). The *hUPII* 5'-flanking DNA of the invention confers urothelial cell-specific expression on heterologous DNA sequences. Additionally, we have found fragments of *hUPII* 5'-flanking DNA
15 (comprising bases about 2028 to about 2239 shown in Figure 1 (SEQ ID NO:1)) that also confer urothelial cell-specific expression on heterologous DNA sequences. The *hUPII* 5'-flanking DNA and fragments thereof disclosed herein can act as a urothelial cell-specific TREs and/or can be included in a urothelial cell-specific TRE. Fragments of *hUPII* 5'-flanking DNA disclosed herein are particularly advantageous
20 for use in vector systems where insert size is limited, such as most viral vector systems.

An urothelial cell-specific TRE, such as one comprising the *hUPII* 5'-flanking DNA sequence or fragment thereof disclosed herein is useful for effecting cell-specific expression, for example, in urothelial cells of urinary tract, thus enabling the
25 directed expression of a desired gene in these cells. For example, vector constructs comprising a heterologous polynucleotide under the transcriptional control of an urothelial cell-specific TRE comprising *hUPII* 5'-flanking DNA can be introduced into bladder cancer cells (particularly transitional cell carcinoma cells) wherein the

heterologous polynucleotide encodes a product which is inhibitory to cell growth, thus controlling the growth of the cancerous cells.

We have also discovered and constructed replication-competent adenovirus vectors which contain an adenovirus gene under transcriptional control of a urothelial cell-specific TRE such that the adenovirus gene is transcribed preferentially in urothelial cells, and have developed methods using these adenovirus vectors. In some preferred embodiments, the adenovirus vectors of this invention comprise at least one adenovirus gene necessary for adenoviral replication, preferably at least one early gene, under the transcriptional control of a TRE. In other preferred embodiments, the adenovirus vectors of this invention comprise co-transcribed first and second genes under control of a urothelial cell-specific TRE, wherein the second gene is under translational control of an internal ribosome entry site (IRES). For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter does not interfere with transcription of the second gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred that the initiation codon of the second gene be removed so that the IRES and second gene are in frame. Alternatively, if the IRES does not contain an initiation codon or if the initiation codon is removed from the IRES, the initiation codon of the second gene is used. By providing for urothelial cell-specific transcription of at least one adenovirus gene required for replication, the invention provides adenovirus vectors that can be used for specific cytotoxic effects due to selective replication and/or selective transcription. This is especially useful in the cancer context, in which targeted cell killing is desirable. This is also useful for targeted cytotoxic effects in other, non-tumor cells, when selective destruction and/or suppression of these cells is desirable. The vectors can also be useful for detecting the presence of cells which permits function of a urothelial cell-specific TRE in, for

example, an appropriate biological (such as clinical) sample. Further, the adenovirus vector(s) can optionally selectively produce one or more proteins of interest in a target urothelial cell by using a urothelial cells-specific TRE.

Adenovirus vectors of the invention replicate and/or express an adenoviral gene operably linked to a urothelial cell-specific TRE preferentially in cells which permits the function of a urothelial cell-specific TRE.

The adenovirus vectors of the present invention comprise a urothelial cell-specific TRE which is functional in a target urothelial cell. The replication preference of these vectors is indicated by comparing the level of replication (i.e., titer) in another, non-urothelial cell type(s). Thus, the invention also uses and takes advantage of what has been considered an undesirable aspect of adenoviral vectors, namely, their replication and possibly concomitant immunogenicity. The probability of runaway infection is significantly reduced due to the urothelial cell-specific requirements for viral replication. Without wishing to be bound by any particular theory, the inventors note that production of adenovirus proteins can serve to activate and/or stimulate the immune system, generally and/or specifically toward target cells producing adenoviral proteins, which can be an important consideration in the cancer context, where patients are often moderately to severely immunocompromised.

The adenovirus vectors of the present invention find particular utility in *inter alia* specific treatment regimens, in which the treatment is highly focused toward bladder cancer which might otherwise be inoperable or untreatable. They are also useful for conferring selective cytotoxicity as well as detection.

General Techniques

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989);

“Oligonucleotide Synthesis” (M.J. Gait, ed., 1984); “Animal Cell Culture” (R.I. Freshney, ed., 1987); “Methods in Enzymology” (Academic Press, Inc.); “Handbook of Experimental Immunology” (D.M. Weir & C.C. Blackwell, eds.); “Gene Transfer Vectors for Mammalian Cells” (J.M. Miller & M.P. Calos, eds., 1987); “Current Protocols in Molecular Biology” (F.M. Ausubel et al., eds., 1987, and periodic updates); “PCR: The Polymerase Chain Reaction”, (Mullis et al., eds., 1994); “Current Protocols in Immunology” (J.E. Coligan et al., eds., 1991).

For techniques related to adenovirus, see, *inter alia*, Felgner and Ringold (1989) *Nature* 337:387-388; Berkner and Sharp (1983) *Nucl. Acids Res.* 11:6003-6020; Graham (1984) *EMBO J.* 3:2917-2922; Bett et al. (1993) *J. Virology* 67:5911-5921; Bett et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:8802-8806.

Definitions

As used herein, a “transcription response element” or “transcriptional regulatory element”, or “TRE” is a polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows that TRE to function. A TRE can comprise an enhancer and/or a promoter. A “transcriptional regulatory sequence” is a TRE.

As used herein, a “urothelial cell-specific transcriptional response element”, or “urothelial cell-specific TRE” is polynucleotide sequence, preferably a DNA sequence, which increases transcription of an operably linked polynucleotide sequence in a host cell that allows a urothelial-specific TRE to function, i.e., a target cell. A variety of urothelial cell-specific TREs are known, are responsive to cellular proteins (transcription factors and/or co-factor(s)) associated with urothelial cells, and comprise at least a portion of a urothelial-specific promoter and/or a urothelial-specific enhancer. Methods are described herein for measuring the activity of a urothelial cell-specific TRE and thus for determining whether a given cell allows a urothelial cell-specific TRE to function.

As described in more detail herein, a urothelial cell-specific TRE can comprise any number of configurations, including, but not limited to, a urothelial cell-specific promoter; a urothelial cell-specific enhancer; a urothelial cell-specific promoter and a urothelial cell-specific enhancer; a urothelial cell-specific promoter and a heterologous enhancer; a heterologous promoter and a urothelial cell-specific enhancer; and multimers of the foregoing. The promoter and enhancer components of a urothelial cell-specific TRE may be in any orientation and/or distance from the coding sequence of interest, as long as the desired urothelial cell-specific transcriptional activity is obtained. Transcriptional activation can be measured in a number of ways known in the art (and described in more detail below), but is generally measured by detection and/or quantitation of mRNA or the protein product of the coding sequence under control of (i.e., operably linked to) the urothelial cell-specific TRE. As discussed herein, a urothelial cell-specific TRE can be of varying lengths, and of varying sequence composition.

A “functional portion” of a urothelial cell-specific TRE is one which confers urothelial cell-specific transcription on an operably linked gene or coding region, such that the operably linked gene or coding region is preferentially expressed in urothelial cells.

A polynucleotide which has or exhibits “urothelial cell-specific activity” increases transcription of an operably linked polynucleotide in a suitable host cell, such as a urothelial cell, preferably a human urothelial cell.

By “transcriptional activation” or an “increase in transcription,” it is intended that transcription is increased above basal levels in the target cell (i.e., urothelial cell) by at least about 2 fold, preferably at least about 5 fold, preferably at least about 10 fold, more preferably at least about 20 fold, more preferably at least about 50 fold, more preferably at least about 100 fold, more preferably at least about 200 fold, even more preferably at least about 400 fold to about 500 fold, even more preferably at least about 1000 fold. Basal levels are generally the level of activity (if any) in a non-

urothelial cell (i.e., a different cell type), or the level of activity (if any) of a reporter construct lacking a urothelial cell-specific TRE as tested in a urothelial, e.g., a bladder carcinoma cell line.

“Replicating preferentially”, as used herein, means that the virus of interest, e.g., an adenovirus replicates more in a urothelial cell than a non-urothelial cell. Preferably, the virus replicates at a significantly higher rate in urothelial cells than non urothelial cells; preferably, at least about 2-fold higher, preferably, at least about 5-fold higher, more preferably, at least about 10-fold higher, still more preferably at least about 50-fold higher, even more preferably at least about 100-fold higher, still more preferably at least about 400- to 500-fold higher, still more preferably at least about 1000-fold higher, most preferably at least about 1×10^6 higher. Most preferably, the vector replicates solely in urothelial cells (that is, does not replicate or replicates at a very low levels in non-urothelial cells).

As used herein, the term “vector” refers to a polynucleotide construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, “cloning vectors” which are designed for isolation, propagation and replication of inserted nucleotides, “expression vectors” which are designed for expression of a nucleotide sequence in a host cell, or a “viral vector” which is designed to result in the production of a recombinant virus or virus-like particle, or “shuttle vectors”, which comprise the attributes of more than one type of vector.

An “adenovirus vector” or “adenoviral vector” (used interchangeably) comprises a polynucleotide construct of the invention. A polynucleotide construct of this invention may be in any of several forms, including, but not limited to, DNA, DNA encapsulated in an adenovirus coat, DNA packaged in another viral or viral-like form (such as herpes simplex, and AAV), DNA encapsulated in liposomes, DNA complexed with polylysine, complexed with synthetic polycationic molecules, conjugated with transferrin, and complexed with compounds such as PEG to immunologically “mask” the molecule and/or increase half-life, and conjugated to a

nonviral protein. Preferably, the polynucleotide is DNA. As used herein, "DNA" includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides. For purposes of this invention, adenovirus vectors are replication-competent in a target cell.

The terms "polynucleotide" and "nucleic acid", used interchangeably herein, refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. These terms include a single-, double- or triple-stranded DNA, genomic DNA, cDNA, RNA, DNA-RNA hybrid, or a polymer comprising purine and pyrimidine bases, or other natural, chemically, biochemically modified, non-natural or derivatized nucleotide bases. The backbone of the polynucleotide can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the polynucleotide can comprise a polymer of synthetic subunits such as phosphoramidates and thus can be a oligodeoxynucleoside phosphoramidate (P-NH₂) or a mixed phosphoramidate- phosphodiester oligomer. Peyrottes et al. (1996) *Nucleic Acids Res.* 24: 1841-8; Chaturvedi et al. (1996) *Nucleic Acids Res.* 24: 2318-23; Schultz et al. (1996) *Nucleic Acids Res.* 24: 2966-73. A phosphorothioate linkage can be used in place of a phosphodiester linkage. Braun et al. (1988) *J. Immunol.* 141: 2084-9; Latimer et al. (1995) *Molec. Immunol.* 32: 1057-1064. In addition, a double-stranded polynucleotide can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer. Reference to a polynucleotide sequence (such as referring to a SEQ ID NO) also includes the complement sequence. As used herein, an "isolated

polynucleotide” means that the polynucleotide is removed from at least one component with which it is naturally associated.

The following are non-limiting examples of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A polynucleotide may comprise modified nucleotides, such as methylated nucleotides and nucleotide analogs, uracyl, other sugars and linking groups such as fluororibose and thioate, and nucleotide branches. The sequence of nucleotides may be interrupted by non-nucleotide components. A polynucleotide may be further modified after polymerization, such as by conjugation with a labeling component. Other types of modifications included in this definition are caps, substitution of one or more of the naturally occurring nucleotides with an analog, and introduction of means for attaching the polynucleotide to proteins, metal ions, labeling components, other polynucleotides, or a solid support. Preferably, the polynucleotide is DNA. As used herein, “DNA” includes not only bases A, T, C, and G, but also includes any of their analogs or modified forms of these bases, such as methylated nucleotides, internucleotide modifications such as uncharged linkages and thioates, use of sugar analogs, and modified and/or alternative backbone structures, such as polyamides.

A polynucleotide or polynucleotide region has a certain percentage (for example, 80%, 85%, 90%, or 95%) of “sequence identity” to another sequence means that, when aligned, that percentage of bases are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1. A preferred alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using

default parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2.

“Under transcriptional control” is a term well understood in the art and indicates that transcription of a polynucleotide sequence, usually a DNA sequence, depends on its being operably (operatively) linked to an element which contributes to the initiation of, or promotes, transcription. “Operably linked” refers to a juxtaposition wherein the elements are in an arrangement allowing them to function.

An “E3 region” (used interchangeably with “E3”) is a term well understood in the art and means the region of the adenoviral genome that encodes the E3 products (discussed herein). Generally, the E3 region is located between about 28583 and 30470 of the adenoviral genome. The E3 region has been described in various publications, including, for example, Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274.

A “portion” of the E3 region means less than the entire E3 region, and as such includes polynucleotide deletions as well as polynucleotides encoding one or more polypeptide products of the E3 region.

An “E1B 19-kDa region” (used interchangeably with “E1B 19-kDa genomic region”) refers to the genomic region of the adenovirus E1B gene encoding the E1B 19-kDa product. According to wild-type Ad5, the E1B 19-kDa region is a 261bp region located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao et al., *Proc. Natl. Acad. Sci. USA*, 89:7742-7746. The present invention encompasses deletion of part or all of the E1B 19-kDa region as well as embodiments wherein the E1B 19-kDa region is mutated, as long as the deletion or mutation lessens or eliminates the inhibition of apoptosis associated with E1B-19kDa.

As used herein, an “internal ribosome entry site” or “IRES” refers to an element that promotes direct internal ribosome entry to the initiation codon, such as ATG, of a cistron (a protein encoding region), thereby leading to the cap-independent

translation of the gene. Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83) and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000).

The present invention encompasses the use of any IRES element which is able to promote direct internal ribosome entry to the initiation codon of a cistron. "Under translational control of an IRES" as used herein means that translation is associated with the IRES and proceeds in a cap-independent manner. Examples of "IRES" known in the art include, but are not limited to IRES obtainable from picornavirus (Jackson et al., 1990, *Trends Biochem Sci* 15(12):477-483); and IRES obtainable from viral or cellular mRNA sources, such as for example, immunoglobulin heavy-chain binding protein (BiP), the vascular endothelial growth factor (VEGF) (Huez et al. (1998) *Mol. Cell. Biol.* 18(11):6178-6190), the fibroblast growth factor 2, and insulin-like growth factor, the translational initiation factor eIF4G, yeast transcription factors TFIID and HAP4. IRES have also been reported in different viruses such as cardiovirus, rhinovirus, aphthovirus, HCV, Friend murine leukemia virus (FrMLV) and Moloney murine leukemia virus (MoMLV). As used herein, "IRES" encompasses functional variations of IRES sequences as long as the variation is able to promote direct internal ribosome entry to the initiation codon of a cistron. In preferred embodiments, the IRES is mammalian. In other embodiments, the IRES is viral or protozoan. In one illustrative embodiment disclosed herein, the IRES is obtainable from encephelomyocarditis virus (ECMV) (commercially available from Novogen, Duke et al. (1992) *J. Virol* 66(3):1602-1609). In another illustrative embodiment disclosed herein, the IRES is from VEGF. Table 6 and Table 7 disclose a variety of IRES sequences useful in the present invention.

In some embodiments, an adenovirus vector comprising co-transcribed first and second genes under transcriptional control of a urothelial-TRE wherein an IRES controls translation of the second gene may exhibit greater specificity for the target cell, ie, bladder cell, than an adenovirus vector comprising a urothelial TRE operably linked to a gene and lacking an IRES. In some embodiments, specificity is conferred

by preferential transcription and/or translation of the first and second genes due to the presence of a urothelial TRE. In other embodiments, specificity is conferred by preferential replication of the adenovirus vectors in target cells due to the urothelial TRE driving transcription of a gene essential for replication.

5 A “multicistronic transcript” refers to an mRNA molecule which contains more than one protein coding region, or cistron. A mRNA comprising two coding regions is denoted a “bicistronic transcript.” The “5’-proximal” coding region or cistron is the coding region whose translation initiation codon (usually AUG) is closest to the 5’-end of a multicistronic mRNA molecule. A “5’-distal” coding region
10 or cistron is one whose translation initiation codon (usually AUG) is not the closest initiation codon to the 5’ end of the mRNA. The terms “5’-distal” and “downstream” are used synonymously to refer to coding regions that are not adjacent to the 5’ end of a mRNA molecule.

 As used herein, “co-transcribed” means that two (or more) coding regions of
15 polynucleotides are under transcriptional control of single transcriptional control element.

 A “gene” refers to a coding region of a polynucleotide. A “gene” may or may not include non-coding sequences and/or regulatory elements.

 “Replication” and “propagation” are used interchangeably and refer to the
20 ability of a polynucleotide construct of the invention to reproduce, or proliferate. This term is well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay, plaque assay, or a one-step growth curve
25 assay.

 As used herein, “cytotoxicity” is a term well understood in the art and refers to a state in which a cell’s usual biochemical or biological activities are compromised (i.e., inhibited). These activities include, but are not limited to, metabolism; cellular

replication; DNA replication; transcription; translation; uptake of molecules.

“Cytotoxicity” includes cell death and/or cytolysis. Assays are known in the art which indicate cytotoxicity, such as dye exclusion, ³H-thymidine uptake, and plaque assays.

5 The term “selective cytotoxicity”, as used herein, refers to the cytotoxicity conferred by an adenovirus vector of the present invention on a cell which allows or induces a urothelial cell-specific TRE to function (a target cell) when compared to the cytotoxicity conferred by an adenoviral vector of the present invention on a cell which does not allow a urothelial cell-specific TRE to function (a non-target cell).
10 Such cytotoxicity may be measured, for example, by plaque assays, by reduction or stabilization in size of a tumor comprising target cells, or the reduction or stabilization of serum levels of a marker characteristic of the tumor cells, or a tissue-specific marker, e.g., a cancer marker.

 In the context of adenovirus, a “heterologous polynucleotide” or
15 “heterologous gene” or “transgene” is any polynucleotide or gene that is not present in wild-type adenovirus. Preferably, the transgene will also not be expressed or present in the target cell prior to introduction by the adenovirus vector. Examples of preferred transgenes are provided below.

 In the context of adenovirus, a “heterologous” promoter or enhancer is one
20 which is not associated with or derived from an adenovirus gene.

 In the context of adenovirus, an “endogenous” promoter, enhancer, or TRE is native to or derived from adenovirus. In the context of promoter, an “inactivation” means that there is a mutation of or deletion in part or all of the of the endogenous promoter, ie, a modification or alteration of the endogenous promoter, such as, for
25 example, a point mutation or insertion, which disables the function of the promoter.

 In the context of a urothelial cell-specific TRE, a “heterologous” promoter or enhancer is one which is derived from a gene other than the gene from which a reference urothelial cell-specific TRE is derived. Examples of a heterologous

promoter with respect to the reference mouse UPII promoter include a viral promoter, an enhancer from a uroplakin gene other than mouse UPII, and a hypoxia-responsive element.

“Suppressing” tumor growth indicates a growth state that is curtailed when compared to growth without contact with, i.e., transfection by, an adenoviral vector described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ³H-thymidine incorporation assay, or counting tumor cells. “Suppressing” tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

As used herein, the terms “neoplastic cells”, “neoplasia”, “tumor”, “tumor cells”, “cancer” and “cancer cells”, (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

A “host cell” includes an individual cell or cell culture which can be or has been a recipient of an adenoviral vector(s) of this invention. Host cells include progeny of a single host cell, and the progeny may not necessarily be completely identical (in morphology or in total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation and/or change. A host cell includes cells transfected or infected *in vivo* or *in vitro* with an adenoviral vector of this invention.

“Replication” and “propagation” are used interchangeably and refer to the ability of an adenovirus vector of the invention to reproduce or proliferate. These terms are well understood in the art. For purposes of this invention, replication involves production of adenovirus proteins and is generally directed to reproduction of adenovirus. Replication can be measured using assays standard in the art and described herein, such as a burst assay or plaque assay. “Replication” and “propagation” include any activity directly or indirectly involved in the process of

virus manufacture, including, but not limited to, viral gene expression; production of viral proteins, nucleic acids or other components; packaging of viral components into complete viruses; and cell lysis.

5 An "ADP coding sequence" is a polynucleotide that encodes ADP or a functional fragment thereof. In the context of ADP, a "functional fragment" of ADP is one that exhibits cytotoxic activity, especially cell lysis, with respect to adenoviral replication. Ways to measure cytotoxic activity are known in the art and are described herein.

10 A polynucleotide that "encodes" an ADP polypeptide is one that can be transcribed and/or translated to produce an ADP polypeptide or a fragment thereof. The anti-sense strand of such a polynucleotide is also said to encode the sequence.

15 An "ADP polypeptide" is a polypeptide containing at least a portion, or region, of the amino acid sequence of an ADP (see, for example, SEQ ID NO:____), and which displays a function associated with ADP, particularly cytotoxicity, more particularly, cell lysis. As discussed herein, these functions can be measured using techniques known in the art. It is understood that certain sequence variations may be used, due to, for example, conservative amino acid substitutions, which may provide ADP polypeptides.

20 A polynucleotide sequence that is "depicted in" a SEQ ID NO means that the sequence is present as an identical contiguous sequence in the SEQ ID NO. The term encompasses portions, or regions of the SEQ ID NO as well as the entire sequence contained within the SEQ ID NO.

25 A "biological sample" encompasses a variety of sample types obtained from an individual and can be used in a diagnostic or monitoring assay. The definition encompasses blood and other liquid samples of biological origin, solid tissue samples such as a biopsy specimen or tissue cultures or cells derived therefrom, and the progeny thereof. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization, or

enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid, and tissue samples.

5 An "individual" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, farm animals, sport animals, rodents, primates, and pets.

10 An "effective amount" is an amount sufficient to effect beneficial or desired results, including clinical results. An effective amount can be administered in one or more administrations. For purposes of this invention, an effective amount of an adenoviral vector is an amount that is sufficient to palliate, ameliorate, stabilize, reverse, slow or delay the progression of the disease state.

A given TRE is "derived from" a given gene if it is associated with that gene in nature.

"Expression" includes transcription and/or translation.

15 As used herein, the term "comprising" and its cognates are used in their inclusive sense; that is, equivalent to the term "including" and its corresponding cognates.

"A," "an" and "the" include plural references unless the context clearly dictates otherwise.

20 ***Human uroplakin transcriptional regulatory sequences***

The present invention provides isolated polynucleotide sequences, derived from the *hUPII* gene, that act to increase the transcription of operably linked polynucleotides in a cell-specific manner. These sequences are of use in controlling the transcription of polynucleotide sequences to which they are operably linked, and thus they may also lend a level of control to the expression of heterologous polynucleotides. These sequences, or a transcriptional regulatory element which they form, can be characterized, in part, by being linked to a polynucleotide sequence, the expression of which they regulate.

25

Accordingly, the present invention encompasses *hUPII* transcriptional control polynucleotides, vectors containing these polynucleotides, host cells containing these polynucleotides, and compositions comprising these polynucleotides. These polynucleotides are isolated and/or produced by chemical and/or recombinant methods, or a combination of these methods. Unless specifically stated otherwise, “polynucleotides” shall include all embodiments of the polynucleotide of this invention. These polynucleotides are useful as probes, primers, in expression systems, and in screening methods as described herein.

It is understood that all polynucleotide embodiments described in this section (“Human uroplakin transcriptional regulatory sequences”) are not depicted in SEQ ID NO:2 (i.e., in any and all of these embodiments, the contiguous nucleotides are not depicted in SEQ ID NO:2 (Fig. 2)). A BLAST search of nucleotides 1 to 2239 (match 1; mismatch -2; gap open 5; gap extension 2) revealed that the sequence spanning from 1 to 2239 of SEQ ID NO:1 shares approximately 81% nucleotide sequence identity over nucleotides 2012 to 2225 of SEQ ID NO:1 with mouse uroplakin II transcriptional regulatory sequences. The longest contiguous identical nucleotide sequence was 16 nucleotides.

We have identified fragments of the human *UPII* (*hUPII*) 5’ untranslated region (UTR) which confer urothelial cell-specific expression on heterologous genes. An approximately 2.2 kb fragment has been isolated (FIG. 1, SEQ ID NO:1) from the 5’ UTR of the *hUPII* gene which confers high level, urothelial cell-specific expression on heterologous genes. Additionally, we have shown that a 1.0 kb fragment (nucleotides 1223 to 2239 of SEQ ID NO:1) of the 5’ UTR sequence of the *hUPII* gene, a 200 bp fragment (nucleotides 2028-2239 of SEQ ID NO:1), a 600 bp fragment (nucleotides 1647-2239 of SEQ ID NO:1), and a 1809 bp fragment (nucleotides 430-2239 of SEQ ID NO:1) confer urothelial cell-specific expression on heterologous genes.

Accordingly, the invention includes an isolated polynucleotide comprising nucleotides about 2028 to about 2239 of SEQ ID NO:1), wherein the polynucleotide exhibits urothelial cell-specific TRE activity (i.e., increases transcription of an operably linked polynucleotide in a suitable host cell, such as a urothelial cells, preferably a human urothelial cell). In other embodiments, the isolated polynucleotide comprises nucleotides about 1223 to about 2239, about 1647 to about 2239, about 430 to about 2239, or about 1 to about 2239 of SEQ ID NO:1, wherein the polynucleotide exhibits urothelial cell specific TRE activity.

In other embodiments, the invention provides an isolated polynucleotide comprising 200 contiguous nucleotides of SEQ ID NO:1 (Fig. 1), wherein the polynucleotide exhibits urothelial cell specific TRE activity. In some embodiments, the contiguous nucleotides are within nucleotides about 2028 to about 2239 of SEQ ID NO:1 (but not depicted in SEQ ID NO:2). In other embodiments, the at least 200 contiguous nucleotides are nucleotides within about 1647 to about 2239, about 1223 to about 2239, about 430 to about 2239, or about 1 to about 2239 of SEQ ID NO:1.

In another embodiment, the invention provides an isolated polynucleotide comprising 200 contiguous nucleotides having at least about 70%, more preferably at least about 75%, 80%, 85%, 90%, 95%, 98% or 99%, sequence identity to a sequence within nucleotides 1 to 2239 of SEQ ID NO:1, with the polynucleotide having urothelial cell-specific TRE activity. In other embodiments, the invention provides an isolated polynucleotide comprising 200 contiguous nucleotides having at least about 70%, more preferably at least about 75%, 80%, 85%, 90%, 95%, 98% or 99%, sequence identity to a sequence within nucleotides 430 to 2239 of SEQ ID NO:1, with the polynucleotide having urothelial cell-specific TRE activity. Various contiguous nucleotides have been provided above, and are included in these embodiments. As noted above, such activity may be found in various lengths of SEQ ID NO:1 (as well as in various regions of SEQ ID NO:1), and may thus also have a longer contiguous nucleotide sequence. In any and all of these embodiments, it is

understood that the contiguous nucleotides are not depicted in SEQ ID NO:2. A preferred alignment program (with parameters) is ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2.

5 An *hUPII* transcriptional regulatory sequence of the invention may be about 100 contiguous nucleotides, about 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 700, 800, 900, 1000, 1200, 1500, 1700, 2000 contiguous nucleotides or larger of the sequence depicted in nucleotides 1 to 2239 of SEQ ID NO:1. Methods for identifying an *hUPII* transcriptional control sequence are routine and well known in the art. For example, overlapping sequences of an *hUPII* transcriptional control sequence can be synthesized and cloned into the vector described in Example 1 to determine *hUPII* transcriptional control activity. Similarly, point mutations can be introduced into the disclosed *hUPII* enhancer sequences using, for example, site-directed mutagenesis or by synthesizing sequences having random nucleotides at one or more predetermined positions and *hUPII* transcriptional control sequence activity determined.

10 As an example of how *hUPII* transcriptional control activity can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested can be inserted into a vector containing a promoter and an appropriate reporter gene encoding a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β -galactosidase (encoded by the *lacZ* gene), luciferase, (encoded by the *luc* gene), alkaline phosphatase, green fluorescent protein, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed are transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative *hUPII* transcriptional regulatory sequence using transfection methods known in the art, such

as calcium phosphate precipitation, electroporation, liposomes (lipofection), and DEAE dextran.

In other embodiments, the isolated polynucleotide (of at least any of the lengths specified above) comprises a region of at least 20 contiguous nucleotides, wherein said region hybridizes under stringent conditions to any of the following regions (i.e., contiguous nucleotides) of SEQ ID NO:1: about 2028 to about 2239; about 1647 to about 2239; about 1223 to about 2239; about 430 to about 2239, about 1 to about 2239, wherein the polynucleotide has urothelial cell specific TRE activity. It is understood that these embodiments also include a polynucleotide (whether single or double stranded) which hybridizes (under stringent conditions) to the corresponding complementary sequence and/or regions of SEQ ID NO:1 as indicated above. In other embodiments, region of the isolated polynucleotide which hybridizes to a sequence of SEQ ID NO:1 is at least any of the following, in terms of contiguous nucleotides: about 25, 30, 50, 75, 100, 150, 250, 300, 350, 400, 450, 500, 750, 1000, 1500. It is understood that a polynucleotide which "hybridizes" to a sequence (region) of SEQ ID NO:1 contains one or more regions of at least 20 contiguous nucleotides which hybridize to a region of SEQ ID NO:1, and that the entire sequence of the polynucleotide which hybridizes to a region of SEQ ID NO:1 need not form a duplex.

In terms of hybridization conditions, the higher the sequence identity required, the more stringent are the hybridization conditions if such sequences are determined by their ability to hybridize to a sequence of SEQ ID NO:1. Accordingly, the invention also includes polynucleotides that are able to hybridize to a sequence comprising at least about 15 contiguous nucleotides (or more, such as about 25, 35, 50, 75 or 100 contiguous nucleotides) of SEQ ID NO:1. The hybridization conditions would be stringent, i.e., 80°C (or higher temperature) and 6M SSC (or less concentrated SSC). Another set of stringent hybridization conditions is 68°C and 0.1 X SSC. For discussion regarding hybridization reactions, see below.

Hybridization reactions can be performed under conditions of different "stringency". Conditions that increase stringency of a hybridization reaction of widely known and published in the art. See, for example, Sambrook et al. (1989) at page 7.52. Examples of relevant conditions include (in order of increasing stringency): incubation temperatures of 25°C, 37°C, 50°C and 68°C; buffer concentrations of 10 X SSC, 6 X SSC, 1 X SSC, 0.1 X SSC (where SSC is 0.15 M NaCl and 15 mM citrate buffer) and their equivalents using other buffer systems; formamide concentrations of 0%, 25%, 50%, and 75%; incubation times from 5 minutes to 24 hours; 1, 2, or more washing steps; wash incubation times of 1, 2, or 15 minutes; and wash solutions of 6 X SSC, 1 X SSC, 0.1 X SSC, or deionized water. An exemplary set of stringent hybridization conditions is 68°C and 0.1 X SSC.

"T_m" is the temperature in degrees Celcius at which 50% of a polynucleotide duplex made of complementary strands hydrogen bonded in anti-parallel direction by Watson-Crick base pairing dissociates into single strands under conditions of the experiment. T_m may be predicted according to a standard formula, such as:

$$T_m = 81.5 + 16.6 \log[X^+] + 0.41 (\%G/C) - 0.61 (\%F) - 600/L$$

where [X⁺] is the cation concentration (usually sodium ion, Na⁺) in mol/L; (%G/C) is the number of G and C residues as a percentage of total residues in the duplex; (%F) is the percent formamide in solution (wt/vol); and L is the number of nucleotides in each strand of the duplex.

Also within the invention are isolated polynucleotides of at least about any of the following lengths, in terms of contiguous nucleotides of SEQ ID NO:1: 15, 20, 30, 100, 150, 200, 250, 300, 400, 450. These polynucleotides may be used, for example, as probes and/or primers.

An *hUPII* transcriptional regulatory sequence may be, or may form part of, an *hUPII* transcriptional regulatory element, or *hUPII*-TRE, which may in turn be

operably linked to a heterologous polynucleotide, i.e., a gene not naturally operably linked to an *hUPII*-TRE. An *hUPII*-TRE would increase expression of an operably linked gene preferentially in those cells which allow an *hUPII*-TRE to function.

5 Examples of heterologous polynucleotides which may be operably linked to an *hUPII*-TRE include, but are not limited to, reporter genes, genes encoding compounds toxic to mammalian cells, genes encoding biological response modifiers, lymphokines, cytokines, cell surface antigens, synthetic genes which direct the synthesis of ribozymes or anti-sense ribonucleotides and genes encoding transcription factors.

10 Marker genes, or reporter genes, which may be employed are known to those skilled in the art and include, but are not limited to, luciferase; aequorin (i.e., green fluorescent protein from *Aequorea victoria*); β -galactosidase, chloramphenicol acetyl transferase; immunologically detectable protein "tags" such as human growth hormone; and the like. See, for example, Current Protocols in Molecular Biology
15 (F.M. Ausubel et al., eds., 1987) and periodic updates. Any assay which detects a product of the reporter gene, either by directly detecting the protein encoded by the reporter gene or by detecting an enzymatic product of a reporter gene-encoded enzyme, is suitable for use in the present invention. Assays include colorimetric, fluorimetric, or luminescent assays or even, in the case of protein tags,
20 radioimmunoassays or other immunological assays.

Toxin genes may include the diphtheria toxin A-chain gene, ricin A-chain gene, *Pseudomonas* exotoxin gene, etc. Maxwell et al. (1987) *Mol. Cell. Biol.* 7:1576; Frankel et al. (1989) *Mol Cell. Biol.* 9:415; Chaudhary et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:4574. Such toxins are known to those skilled in the art.

25 Other toxin genes may include mutated or truncated forms of naturally-occurring proteins which competitively or non-competitively inhibit the correct functioning of the naturally-occurring forms and which thereby may kill the cell. Alternatively, a toxin gene may comprise a gene that, when expressed, causes apoptosis.

Lymphokines and cytokines are known in the art and include, but are not limited to, interleukins, interferons, colony-stimulating factors, etc.

Cell surface antigens include those which are not normally expressed on the surface of a given cell, and result in enhance immunocytotoxicity or immune reactivity toward the cell.

Synthetic genes which direct the synthesis of ribozymes or anti-sense ribonucleotides may also be operably linked to an *hUPII* transcriptional regulatory sequence. Antisense RNA and DNA molecules and ribozymes may function to inhibit translation of a protein. S. T. Crooke and B. Lebleu, eds. *Antisense Research and Applications* (1993) CRC Press; and *Antisense RNA and DNA* (1988) D.A. Melton, Ed. Cold Spring Harbor Laboratory Cold Spring Harbor, NY. Anti-sense RNA and DNA molecules act to directly block the translation of mRNA by binding to targeted mRNA and preventing protein translation. Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. The mechanism of ribozyme action involves sequence specific interaction of the ribozyme molecule to complementary target RNA, followed by an endonucleolytic cleavage. Within the scope of the invention are engineered hammerhead or other motif ribozyme molecules that specifically and efficiently catalyze endonucleolytic cleavage of RNA sequences.

Compositions comprising an *hUPII* transcriptional regulatory polynucleotide as well as compositions comprising an *hUPII*-TRE operably linked to a heterologous polynucleotide are encompassed by this invention. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable excipient. Accordingly, the invention also provides compositions of these polynucleotides, including compositions comprising these polynucleotides and a pharmaceutical excipient, as well pharmaceutical compositions comprising these vectors. Pharmaceutical excipients are well known in the art and need not be described in detail herein. See, for example, *Remington: The Science and Practice of*

Pharmacy (19th edition, 1995), Gennaro, ed. When these compositions are used for other purposes, such as detection (i.e., hybridization, amplification (i.e., PCR), and testing for function (i.e., transcription assay), these compositions may comprise suitable agents such as a buffer or a physiologically acceptable excipient.

5 Also included in the invention are kits comprising any one or more of the polynucleotides described herein in suitable packaging. These kits can be used, for example, for detection. The kits may optionally also contain additional components, such as buffers, capture reagents, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

10 ***Preparation of hUPII transcriptional regulatory polynucleotides of the invention***

 The *hUPII* transcriptional regulatory polynucleotides of this invention can be obtained using chemical synthesis, recombinant methods, or PCR.

 Methods of chemical polynucleotide synthesis are well known in the art and need not be described in detail herein. One of skill in the art can use the sequences
15 provided herein and a commercial DNA synthesizer to produce a desired DNA sequence.

 For preparing *hUPII* transcriptional regulatory polynucleotides using recombinant methods, a polynucleotide comprising a desired sequence can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable
20 host cell for replication and amplification. Polynucleotides may be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, f-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the
25 host cell genome. The polynucleotide so amplified can be isolated from the host cell by methods well known within the art. See, e.g., Sambrook et al. (1989).

 Alternatively, PCR allows reproduction of DNA sequences. PCR technology is well known in the art and is described in U.S. Pat. Nos. 4,683,195, 4,800,159,

4,754,065 and 4,683,202, as well as *PCR: The Polymerase Chain Reaction*, Mullis et al. eds., Birkauswer Press, Boston (1994).

RNA can be obtained by using the isolated DNA in an appropriate vector and inserting it into a suitable host cell. When the cell replicates and the DNA is transcribed into RNA, the RNA can then be isolated using methods well known to those of skill in the art, as set forth in Sambrook et al., (1989), for example. RNA can also be obtained through in vitro reactions. An *hUPII* transcriptional regulatory polynucleotide can be inserted into a vector that contains appropriate transcription promoter sequences. Commercially available RNA polymerases will specifically initiate transcription at their promoter sites and continue the transcription process through the adjoining DNA polynucleotides. Placing *hUPII* transcriptional regulatory polynucleotides between two such promoters allows the generation of sense or antisense strands of *hUPII* transcriptional regulatory RNA sequence.

Cloning and expression vectors comprising an hUPII transcriptional regulatory polynucleotide

The present invention further includes a variety of vectors (i.e., cloning and expression vectors) having cloned therein *hUPII* transcriptional regulatory polynucleotide(s). These vectors can be used for expression of recombinant polypeptides as well as a source of *hUPII* transcriptional regulatory polynucleotides. Cloning vectors can be used to obtain replicate copies of the *hUPII* transcriptional regulatory polynucleotides they contain, or as a means of storing the polynucleotides in a depository for future recovery. Expression vectors (and host cells containing these expression vectors) can be used to obtain polypeptides produced from the polynucleotides they contain. They may also be used where it is desirable to express polypeptides, encoded by an operably linked polynucleotide, in an individual, such as for eliciting an immune response via the polypeptide(s) encoded in the expression vector(s). Suitable cloning and expression vectors include any known in the art, e.g.,

those for use in bacterial, mammalian, yeast and insect expression systems. Specific vectors and suitable host cells are known in the art and need not be described in detail herein. For example, see Gacesa and Ramji, *Vectors*, John Wiley & Sons (1994).

Cloning and expression vectors typically contain a selectable marker (for example, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector), although such a marker gene can be carried on another polynucleotide sequence co-introduced into the host cell. Only those host cells into which a selectable gene has been introduced will survive and/or grow under selective conditions. Typical selection genes encode protein(s) that (a) confer resistance to antibiotics or other toxins substances, e.g., ampicillin, neomycin, methotrexate, etc.; (b) complement auxotrophic deficiencies; or (c) supply critical nutrients not available from complex media. The choice of the proper marker gene will depend on the host cell, and appropriate genes for different hosts are known in the art. Cloning and expression vectors also typically contain a replication system recognized by the host.

Suitable cloning vectors may be constructed according to standard techniques, or may be selected from a large number of cloning vectors available in the art. While the cloning vector selected may vary according to the host cell intended to be used, useful cloning vectors will generally have the ability to self-replicate, may possess a single target for a particular restriction endonuclease, and/or may carry genes for a marker that can be used in selecting clones containing the vector. Suitable examples include plasmids and bacterial viruses, e.g., pUC18, pUC19, Bluescript (e.g., pBS SK+) and its derivatives, mp18, mp19, pBR322, pMB9, ColE1, pCR1, RP4, phage DNAs, and shuttle vectors such as pSA3 and pAT28. These and many other cloning vectors are available from commercial vendors such as BioRad, Strategene, and Invitrogen. The Examples provided herein also provide examples of cloning vectors.

Expression vectors generally are replicable polynucleotide constructs that contain a polynucleotide encoding a polypeptide of interest. The polynucleotide encoding the polypeptide of interest is operably linked to suitable transcriptional

controlling elements, such as promoters, enhancers and terminators. For expression (i.e., translation), one or more translational controlling elements are also usually required, such as ribosome binding sites, translation initiation sites, and stop codons. These controlling elements (transcriptional and translational) may be derived from *hUPII* polynucleotides (e.g., the *hUPII* gene), or they may be heterologous (i.e., derived from other genes and/or other organisms). A polynucleotide sequence encoding a signal peptide can also be included to allow a polypeptide, encoded by an operably linked polynucleotide, to cross and/or lodge in cell membranes or be secreted from the cell. A number of expression vectors suitable for expression in eukaryotic cells including yeast, avian, and mammalian cells are known in the art. Examples of mammalian expression vectors contain both prokaryotic sequence to facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. Examples of mammalian expression vectors suitable for transfection of eukaryotic cells include the pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pRSVneo, and pHyg derived vectors. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEB, pREP derived vectors) can be used for expression in mammalian cells. Examples of expression vectors for yeast systems, include YEP24, YIP5, YEP51, YEP52, YES2 and YRP17, which are cloning and expression vehicles useful for introduction of constructs into *S. cerevisiae*. Broach et al. (1983) *Experimental Manipulation of Gene Expression*, ed. M. Inouye, Academic Press. p. 83. Other common vectors, such as YEP13 and the Sikorski series pRS303-306, 313-316, 423-426 can also be used. Vectors pDBV52 and pDBV53 are suitable for expression in *C. albicans*. Baculovirus expression vectors for expression in insect cells include pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors and pBlueBac-derived vectors.

A vector comprising an *hUPII* transcriptional regulatory polynucleotide can be introduced into a host cell and/or a target cell by any of a number of appropriate

means, including electroporation, transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; and infection (where the vector is an infectious agent, such as vaccinia virus). The choice of means of introducing vectors or *hUPII* transcriptional regulatory polynucleotides will often depend on the host cell or target cell. A vector comprising an *hUPII* transcriptional regulatory polynucleotide can also be delivered to a host cell and/or a target cell in the form of a delivery vehicle, described below.

Delivery Vehicles Containing an hUPII transcriptional regulatory polynucleotide

The present invention also provides delivery vehicles suitable for delivery of an *hUPII* transcriptional regulatory polynucleotide into cells (whether *in vivo*, *ex vivo*, or *in vitro*). Generally, an *hUPII* transcriptional regulatory sequence will be operably linked to a heterologous polynucleotide. An *hUPII* transcriptional regulatory polynucleotide can be contained within a cloning or expression vector, as described above, or within a viral vector. These vectors (especially expression vectors) can in turn be manipulated to assume any of a number of forms which may, for example, facilitate delivery to and/or entry into a target cell. Delivery of the polynucleotide constructs of the invention to eukaryotic cells, particularly to mammalian cells, more particularly to bladder cells, can be accomplished by any suitable art-known method. Delivery can be accomplished *in vivo*, *ex vivo*, or *in vitro*.

The invention provides methods and compositions for transferring such expression constructs into cells, especially *in vivo* for treatment of bladder tumors.

Delivery vehicles suitable for incorporation of an *hUPII* transcriptional regulatory sequence of the present invention for introduction into a host cell include non-viral vehicles and viral vectors. Verma and Somia (1997) *Nature* 389:239-242.

Non-viral vehicles

A wide variety of non-viral vehicles for delivery of *hUPII* transcriptional regulatory polynucleotides of the present invention are known in the art and are encompassed in the present invention. An *hUPII* transcriptional regulatory polynucleotide can be delivered to a cell as naked DNA (U.S. Patent No. 5,692,622; WO 97/40163). Alternatively, an *hUPII* transcriptional regulatory polynucleotide can be delivered to a cell associated in a variety of ways with a variety of substances (forms of delivery) including, but not limited to cationic lipids; biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria. A delivery vehicle may take the form of a microparticle. Mixtures or conjugates of these various substances can also be used as delivery vehicles. An *hUPII* transcriptional regulatory polynucleotide can be associated with these various forms of delivery non-covalently or covalently.

One non-viral gene transfer vehicle suitable for use in the present invention is physical transfer of a polynucleotide in cationic lipids, which can take the form of liposomes. Reviewed in Mahato et al. (1997) *Pharm. Res.* 14:853-859. Liposomal preparations include, but are not limited to, cytofectins, multilamellar vesicles and unilamellar vesicles. Several commercial liposomal preparations are available for the delivery of DNA and RNA to cells, including but not limited to, Lipofectin™, Lipofectamine™, and DOTAP™.

Derivatized liposomes can be used as carriers of *hUPII* transcriptional regulatory polynucleotides. Immunoliposomes are derivatized liposomes which contain on their surface specific antibodies which bind to surface antigens on specific cell types, thus targeting these liposomes to particular cell types. Wang and Huang (1987) *Proc. Natl. Acad. Sci. (U.S.A.)* 84:7851; and Trubetskoy et al. (1992) *Biochem. Biophys. Acta* 1131:311. Other types of derivatization include modification

of the liposomes to include ligands which bind to receptors on particular cell types, or receptors which bind specifically to cell surface molecules.

Lipopolyamine can be used as a reagent to mediate transfection itself, without the necessity of any additional phospholipid to form liposomes. Behr et al. (1989) *Proc. Natl. Acad. Sci. (U.S.A.)* 86:6982.

Other lipid-based delivery vehicles are known and have been described, and can be used in the present invention. For example, U.S. Patent No. 5,705,385 discloses lipid-nucleic acid particles for gene delivery via formation of hydrophobic lipid-nucleic acid complexes. The complexes are charge-neutralized. Formation of these complexes in either detergent-based or organic solvent-based systems, followed by removal of the detergent or organic solvent, leads to particle formation.

Polypeptide gene delivery vehicles include polyamino acids such as polylysine, and various naturally occurring polypeptides such as gelatin, and conjugates of these with other macromolecules.

Low molecular weight polylysine (PL) and other polycations can be used as carriers to promote DNA-mediated transfection into cultured mammalian cells. Zhou et al. (1991) *Biochem. Biophys. Acta* 1065:1068 reports synthesis of a polylysine-phospholipid conjugate, a lipopolylysine comprising PL linked to N-glutarylphosphatidylethanolamine, which reportedly increases the transfection efficiency of DNA.

Polylysine molecules conjugated to asialoorosomucoid ("ASOR") or transferrin can be used for target-specific delivery of associated polynucleotides to cells which express the appropriate receptor (i.e., asialoglycoprotein receptor or transferrin receptor, respectively). Such conjugates have been described. Wilson et al. (1992) *J. Biol. Chem.* 267:963; WO92/06180; WO92/05250; WO91/17761; Wagner et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:3410; Zenke et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87:3655; and WO92/13570.

Polypeptide delivery vehicles include those which form microspheres, as described. WO 96/00295. Polypeptide microspheres can comprise polypeptide alone or mixtures of polypeptides with other macromolecules, for example chondroitin sulfate. The polypeptides may be crosslinked, as described. WO 96/40829. In addition, a targeting moiety can be incorporated into such polypeptide delivery vehicles.

Microparticles for delivery of polynucleotides into cells are known and can be used to deliver *hUPII* transcriptional regulatory polynucleotides to a cell. Microparticles generally comprise a polynucleotide and a substance which facilitates entry into a cell. These include, for example, polymeric cations, complexes of hydrophobized, positively charged biocompatible polymer and a lipoprotein (U.S. Patent No. 5,679,559); complexes of a receptor ligand and a polycation (U.S. Patent No. 5,635,383); polycation conjugated with polyalkylene glycol or a polysaccharide (WO 96/21036); a complex between a fusion protein comprising a domain which specifically binds an *hUPII* transcriptional regulatory polynucleotide and a domain which targets a particular cell type (EP 753,069); chylomicrons (Hara et al. (1997) *Proc. Natl. Acad. Sci. USA* 94:14547-14552); metal particles such as tungsten and gold (Zelenin et al. (1997) *FEBS Letters* 414:319-322; and chitosan-based compounds (WO 97/42975).

Other types of carriers include covalently bound conjugates consisting of oligonucleotides in disulfide linkage to a targeting agent that promotes transport across cell membranes (WO 91/14696); artificial viral envelopes (Schreier et al. (1995) *J. Molec. Recognition* 8:59-62; and Chander and Schreier (1992) *Life Sci.* 50:481-489; and bacteria, for example *Salmonella* (Pawelek et al. (1997) *Cancer Res.* 57:4537-4544); and *Listeria monocytogenes* (Dietrich et al. (1998) *Nature Biotech.* 16:181-185).

The delivery vehicles of the present invention can include one or more targeting molecules incorporated into or attached to the vehicle. Targeting molecules

include any molecule that binds specifically to a target cell type. This can be any type of molecule for which a specific binding partner exists. The term "specific binding partner" as used herein intends a member of a pair of molecules that interact by means of specific non-covalent interactions that depend on the three-dimensional structures of the molecules involved. Preferably, the specific binding partner is expressed only on the target cell type. Examples of targeting molecules which may be used are hormones, antibodies, cell adhesion molecules, saccharides, drugs, and neurotransmitters.

Compositions comprising an *hUPII* transcriptional regulatory polynucleotide in a delivery vehicle are encompassed by this invention. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable excipient. Accordingly, the invention also provides compositions of these vectors, including compositions comprising these vectors and a pharmaceutical excipient, as well pharmaceutical compositions comprising these vectors. Pharmaceutical excipients are well known in the art and need not be described in detail herein. See, for example, *Remington: The Science and Practice of Pharmacy* (19th edition, 1995), Gennaro, ed.

An *hUPII* transcriptional regulatory polynucleotide can be inserted into a non-viral vector for delivery into a cell, as described above. Included in the non-viral vector category are prokaryotic plasmids and eukaryotic plasmids, as described above. One skilled in the art will appreciate that a wide variety of such vectors are known, are readily available, and can be used in the present invention. An *hUPII* transcriptional regulatory polynucleotide inserted into a non-viral vector can be delivered to a cell with the help of any of the above-described vehicles, as well as direct injection of the polynucleotide, or other types of delivery methods. The above-described delivery vehicles can also be used to delivery an *hUPII* transcriptional regulatory polynucleotide inserted into a viral vector.

Preparation of non-viral vehicles comprising an hUPII transcriptional regulatory polynucleotide

Preparation of liposomes for transfer of polynucleotides can be carried out as described by various investigators (Wang and Huang (1987) *Biochem. Biophys. Res. Commun.* 147:980; Wang and Huang (1989) *Biochemistry* 28:9508; Litzinger and Huang (1992) *Biochem. Biophys. Acta* 1113 201; Gao and Huang (1991) *Biochem. Biophys. Res. Commun.* 179:280; Felgner WO91/17424; WO91/16024).

The preparation of other types of non-viral vehicles is known in the art and has been described. For example, preparation of polylysine delivery vehicles has been described by Zhou et al. (1991) *Biochem. Biophys. Acta* 1065:1068. Methods for preparation of microparticles of various compositions have also been described (see publications cited above) and are known in the art.

Introduction of targeting molecules into the non-viral vehicles of the present invention can be carried out by any known means, including incorporation into a cationic lipid vehicle or a microsphere or a microparticle; by direct chemical conjugation with a macromolecule of which the delivery vehicle is comprised, or any other known methods.

Viral vectors

An *hUPII* transcriptional regulatory polynucleotide can be inserted into a viral vector. Viral vectors include, but are not limited to, DNA viral vectors such as those based on adenoviruses, herpes simplex virus, poxviruses such as vaccinia virus, and parvoviruses, including adeno-associated virus; and RNA viral vectors, including, but not limited to, the retroviral vectors. Retroviral vectors include murine leukemia virus, and lentiviruses such as human immunodeficiency virus. Naldini et al. (1996) *Science* 272:263-267.

Replication-defective retroviral vectors harboring an *hUPII* polynucleotide sequence as part of the retroviral genome can be used. Such vectors have been

described in detail. (Miller et al. (1990) *Mol. Cell Biol.* 10:4239; Kolberg, R. (1992) *J. NIH Res.* 4:43; Cornetta et al. (1991) *Hum. Gene Ther* 2:215). The major advantages of retroviral vectors for gene therapy are: the high efficiency of gene transfer into replicating cells, the precise integration of the transferred genes into cellular DNA, and the lack of further spread of the sequences after gene transduction.

Representative examples of retroviral gene delivery vehicles that may be utilized within the context of the present invention include, for example, those described in EP 415,731; WO 90/07936; WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218; Vile and Hart, *Cancer Res.* 53:83-88, 1993; Vile and Hart, *Cancer Res.* 53:962-967, 1993; Ram et al., *Cancer Res.* 53:83-88, 1993; Takamiya et al., *J. Neurosci. Res.* 33: 493-503, 1992; Baba et al., *J. Neurosurg* 79:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO 91/02805).

Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include psi Crip, psi cre, psi 2 and psi Am. Retroviruses have been used to delivery a variety of polynucleotides into many different cell types. See, for example, Kay et al. (1992) *Human Gene Therapy* 3:641-647; Dai et al. (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al. (1993) *J. Immunol.* 150:4104-4115.

Adenoviral vectors can also be used for delivery of *hUPII* transcriptional regulatory polynucleotides. Rosenfeld et al. (1992) *Cell* 68:143. Accordingly, the invention provides an adenovirus vector comprising any human uroplakin II transcriptional regulatory sequence described herein. The sequence may be operably linked to an adenovirus gene and/or a transgene. Certain adenoviral vector embodiments of the invention are further discussed in a separate section. It is understood that, with respect to the *hUPII* transcriptional regulatory polynucleotides described herein, any adenoviral vector containing any of these sequences is

encompassed by the invention. Major advantages of adenovirus vectors are their potential to carry large insert polynucleotide sequences, very high viral titres, ability to infect non-replicating cells, and suitability for infecting tissues *in situ*.

5 For the purposes of this invention, the adenoviral vectors can be replication competent or replication defective, depending on the desired outcome of infection with virus.

10 In general, replication-defective adenovirus gene transfer systems are based upon recombinant, engineered adenovirus which is rendered replication-incompetent by deletion of a portion of its genome, such as E1 or E3, and yet still retains its competency for infection. Berkner et al. (1988) *BioTechniques* 6:616; Rosenfeld et al. (1991) *Science* 252:431-434; Rosenfeld et al. (1992) *Cell* 68:143-155. Relatively large foreign proteins can be expressed when additional deletions are made in the adenovirus genome. For example, adenoviruses deleted in both E1 and E3 regions are capable of carrying up to 10 kb of foreign DNA and can be grown to high titers in 293 cells. Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7, etc.) are well known to those skilled in the art.

20 Another viral vector system useful for delivery of an *hUPII* transcriptional regulatory polynucleotide is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. Muzyczka et al. (1992) *Curr. Topics Microbiol. Immunol.* 158:97-129. AAV as a delivery vehicle for an *hUPII* transcriptional regulatory polynucleotide can be constructed and introduced into cells by any means known in the art, including the methods described in U.S. Patent No. 5,658,785.

25 In addition to the viral vectors describe above, numerous other viral vectors systems may also be utilized as a gene delivery vehicle. Representative examples of such gene delivery vehicles include viruses such as pox viruses, such as canary virus

or vaccinia virus (Fisher-Hoch et al., *PNAS* 86:317-321, 1989; Flexner et al., *Ann N.Y. Acad. Sci.* 569:86-103, 1989; Flexner et al., *Vaccine* 8:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, 5,017,487 and 5,656,465; WO 89/01973); SV40 (Mulligan et al., *Nature* 277:108-114, 1979; influenza virus (Luytjes et al., *Cell* 59:1107-1113, 1989; McMichael et al., *N. Eng. J. Med.* 309:13-17, 1983; and Yap et al., *Nature* 273:238-239, 1978; herpes (Kit, *Adv. Exp. Med. Biol.* 215:219-236 1989; U.S. Patent No. 5,288,641); HIV (Poznansky, *J. Virol.* 65:532-536, 1991); measles (EP 0 440,219); Semliki Forest Virus, and coronavirus, as well as other viral systems (e.g., EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic (defective), replication competent virus (e.g., Overbaugh et al., *Science* 239:906-910, 1988), and nevertheless induce cellular immune responses, including CTL.

Viral vectors comprising an *hUPII* transcriptional regulatory polynucleotide can be targeted to a particular cell type for more efficient delivery of an *hUPII* transcriptional regulatory polynucleotide, for example, to a neoplastic bladder urothelial cell. For example, a viral vector can comprise, in addition to an *hUPII* transcriptional regulatory polynucleotide, a polynucleotide encoding one member of a specific binding pair which inserts into the viral envelope or capsid and which targets the viral particle to a cell having the complementary member of the specific binding pair on its surface. WO 95/26412. Alternatively, the surface of a viral particle can be covalently modified to target it to a particular cell. WO 92/06180; WO 92/05266.

Viral vectors can be so constructed that they contain regulatable control elements which are controlled, for example, by tetracycline. WO 97/20463.

Virus-based vectors can also be used to deliver an *hUPII* transcriptional regulatory polynucleotide. These include retrotransposon vectors (U.S. Patent No. 5,354,674) and synthetic vectors (WO 94/20608; WO 96/26745).

Viral vectors comprising an IRES

In one aspect of the present invention, the adenovirus vectors comprise co-transcribed first and second genes under control of a urothelial cell-specific TRE, such a hUPII TRE, wherein the second gene is under translational control of an internal ribosome entry site (IRES). IRES elements were first discovered in picornavirus mRNAs (Jackson RJ, Howell MT, Kaminski A (1990) *Trends Biochem Sci* 15(12):477-83) and Jackson RJ and Kaminski, A. (1995) *RNA* 1(10):985-1000). The present invention provides improved adenovirus vectors comprising co-transcribed first and second genes under transcriptional control of a heterologous, target cell-specific TRE, and wherein the second gene (i.e., coding region) is under translational control of an internal ribosome entry site (IRES). Any IRES may be used in the adenovirus vectors of the invention, as long as they exhibit requisite function in the vectors. Example of IRES which can be used in the present invention include those provided in Table 6 and referenced in Table 7. Examples of IRES elements include the encephelomyocarditis virus (EMCV) which is commercially available from Novagen (Duke et al. (1992) *J. Virol* 66(3):1602-9) the sequence for which is depicted in Table 6 (SEQ ID NO: ____). Another example of an IRES element disclosed herein is the VEGF IRES (Huez et al. (1998) *Mol Cell Biol* 18(11):6178-90). This IRES has short segment and the sequence is depicted in Table 6 (SEQ ID NO: ____).

The IRES promotes direct internal ribosome entry to the initiation codon of a downstream cistron, leading to cap-independent translation. Thus, the product of a downstream cistron can be expressed from a bicistronic (or multicistronic) mRNA, without requiring either cleavage of a polyprotein or generation of a monocistronic mRNA. Therefore, in one illustrative embodiment of the present invention, an adenovirus vector comprising E1B under translational control of an IRES allows translation of E1B from a bicistronic E1A-E1B mRNA under control of a target cell-

specific TRE. Figure 7 provides a schematic representation of adenovirus constructs of the present invention.

Internal ribosome entry sites are approximately 450 nucleotides in length and are characterized by moderate conservation of primary sequence and strong conservation of secondary structure. The most significant primary sequence feature of the IRES is a pyrimidine-rich site whose start is located approximately 25 nucleotides upstream of the 3' end of the IRES. See Jackson *et al.* (1990).

Three major classes of picornavirus IRES have been identified and characterized: (1) the cardio- and aphthovirus class (for example, the encephelomyocarditis virus, Jang *et al.* (1990) *Gene Dev* 4:1560-1572); (2) the entero- and rhinovirus class (for example, polioviruses, Borman *et al.* (1994) *EMBO J.* 13:314903157); and (3) the hepatitis A virus (HAV) class, Glass *et al.* (1993) *Virology* 193:842-852). For the first two classes, two general principles apply. First, most of the 450-nucleotide sequence of the IRES functions to maintain particular secondary and tertiary structures conducive to ribosome binding and translational initiation. Second, the ribosome entry site is an AUG triplet located at the 3' end of the IRES, approximately 25 nucleotides downstream of a conserved oligopyrimidine tract. Translation initiation can occur either at the ribosome entry site (cardioviruses) or at the next downstream AUG (entero/rhinovirus class). Initiation occurs at both sites in aphthoviruses.

HCV and pestiviruses such as bovine viral diarrhea virus (BVDV) or classical swine fever virus (CSFV) have 341 nt and 370 nt long 5'-UTR respectively. These 5'-UTR fragments form similar RNA secondary structures and can have moderately efficient IRES function (Tsukiyama-Kohara *et al.* (1992) *J. Virol.* 66:1476-1483; Frolov I *et al.*, (1998) (RNA) 4:1418-1435). Table 6 depicts the 5'-UTR region from HCV genome sequence (GenBank accession D14853).

Leishmania RNA virus 1 (LRV1) is a double-stranded RNA virus. Its 128 nt long 5'-UTR has IRES activity to facilitate the cap-independent translation, Maga *et*

al.,(1995) *Mol Cell Biol* 15:4884-4889). This fragment also forms conserved stemloop secondary structure and at least the front part is essential.

Recent studies showed that both Friend-murine leukemia virus (MLV) 5'-UTR and rat retrotransposon virus-like 30S (VL30) sequences contain IRES structure of retroviral origin (Torrent et al. (1996) *Hum Gene Ther* 7:603-612). These fragments are also functional as packing signal when used in retroviruse derived vectors. Studies of avian reticuloendotheliosis virus type A (REV-A) show that its IRES maps downstream of the packaging/dimerization (E/DLS) sequence and the minimal IRES sequence appears to be within a 129 nt fragment (452-580) of the 5' leader, immediately upstream of the gag AUG codon (Lopez-Lastra et al. (1997) *Hum Gene Ther* 8:1855-1865).

In eukaryotic cells, translation is normally initiated by the ribosome scanning from the capped mRNA 5' end, under the control of initiation factors. However, several cellular mRNAs have been found to be with IRES structure to mediate the cap-independent translation (van der Velde, et al. (1999) *Int J Biochem Cell Biol.* 31:87-106). Examples are immunoglobulin heavy-chain binding protein (BiP) (Macejak et al. (1991) *Nature* 353:90-94), antennapedia mRNA of *Drosophila* (Oh et al. (1992) *Gene and Dev* 6:1643-1653), fibroblast growth factor-2 (FGF-2) (Vagner et al. (1995) *Mol Cell Biol* 15:35-44), platelet-derived growth factor B (PDGF-B) (Bernstein et al. (1997) *J Biol Chem* 272:9356-9362), insulin-like growth factor II (Teerink et al. (1995) *Biochim Biophys Acta* 1264:403-408), and the translation initiation factor eIF4G (Gan et al. (1996) *J Biol Chem* 271:623-626). Table 6 depicts the 5'-noncoding region for BiP and PDGF. Recently, vascular endothelial growth factor (VEGF) was also found to have IRES element (Stein et al. (1998) *Mol Cell Biol* 18:3112-3119; Huez et al. (1998) *Mol Cell Biol* 18:6178-6190).

Apart from the oligopyrimidine tract, nucleotide sequence *per se* does not appear to be important for IRES function. Without wishing to be bound by theory, a possible explanation for the function of an IRES is that it forms secondary and/or

tertiary structures which orient particular single-stranded regions of its sequence in a three-dimensional configuration that is conducive to interaction with a mammalian ribosome (either ribosomal protein and/or ribosomal RNA components) and/or initiation factor(s) and/or RNA binding proteins which interact with ribosomes and/or initiation factors. It is also possible that the three-dimensional structure of the IRES is determined or stabilized by one or more RNA-binding proteins. Thus it is possible to devise synthetic IRES sequences having similar single-stranded regions in a similar three-dimensional configuration.

In certain cases, one or more *trans*-acting cellular proteins may be required for IRES function. For example, the HAV and entero/rhinovirus IRESes function inefficiently *in vitro* in reticulocyte lysates. Supplementation of a reticulocyte lysate with a cytoplasmic extract from HeLa, Krebs II ascites, or L-cells restores activity of entero/rhinovirus IRESes. See, for example, Brown *et al.* (1979) *Virology* 97:396-405; and Dorner *et al.* (1984) *J. Virol.* 50:507-514. Activity of the HAV IRES *in vitro* is stimulated by liver cytoplasmic extracts. Glass *et al.* (1993) *Virology* 193:1047-1050. These observations indicate that cell-specific translational regulation can be achieved through the use of a cell-specific IRES. Furthermore, coordinated cell-specific transcriptional and translational regulatory elements can be included in a vector to further increase cell specificity of viral replication. For example, the combination of an AFP-TRE and a HAV-IRES can be used to direct preferential replication of a vector in hepatic cells. Thus, in one illustrative embodiment, a vector comprises an AFP-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES. In another illustrative embodiment, the vector comprises a probasin-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES. In yet another illustrative embodiment, a vector comprises a CMV-TRE regulating the transcription of a bicistronic E1A-E1B mRNA in which E1B translation is regulated by an ECMV IRES.

Examples of IRES which can be used in the present invention include those provided in Table 6 and Table 7. In order to test for an IRES sequence which may be used in the present invention, a test vector is produced having a reporter gene, such as luciferase, for example, placed under translational control of an IRES to be tested. A
5 desired cell type is transfected with the vector containing the desired IRES-reporter gene and an assay is performed to detect the presence of the reporter gene. In one illustrative example, the test vector comprises a co-transcribed chloramphenicol transferase (CAT) and luciferase encoding gene transcriptionally driven by a CMV promoter wherein the luciferase encoding gene is translationally driven by an IRES to
10 be tested. Host cells are transiently transfected with the test vector by means known to those of skill in the art and assayed for the presence of luciferase.

IRES may be prepared using standard recombinant and synthetic methods known in the art, and as described in the Examples. For cloning convenience, restriction sites may be engineered into the ends of the IRES fragments to be used.

15
Preparation of viral vectors comprising an hUPII transcriptional regulatory polynucleotide

The basic technique of inserting genes into viruses are known to the skilled artisan and involve, for example, recombination between the viral polynucleotide sequences flanking a polynucleotide in a donor plasmid and homologous sequences
20 present in the parental virus. Mackett et al. (1982) *Proc. Natl. Acad. Sci. USA* 79:7415-7419. For example, a unique restriction site that is naturally present or artificially inserted in the parental viral vector can be used to insert a polynucleotide flanked by the same restriction site as in the viral vector.

25 A DNA virus can be constructed as follows. First, the polynucleotide sequence to be inserted into the virus can be placed into a plasmid, e.g., an *E. coli* plasmid construct, into which a polynucleotide homologous to a section of the polynucleotide such as that of the virus has been inserted. Separately the

polynucleotide sequence to be inserted is ligated to a promoter. The promoter-gene linkage is positioned in the plasmid construct so that the promoter-gene linkage is flanked on both ends by polynucleotide sequences homologous to a polynucleotide sequence flanking a region of viral DNA which is the desired insertion region. The resulting plasmid construct is then amplified by growth within *E. coli* bacteria and isolated. Preferably, the plasmid also contains an origin of replication such as the *E. coli* origin of replication, and a marker such as an antibiotic resistance gene for selection and propagation in *E. coli*.

Second, the isolated plasmid containing the polynucleotide sequence to be inserted is transfected into a cell culture, e.g., chick embryo fibroblasts, along with the virus. Recombination between homologous DNA in the plasmid and the viral genome respectively results in a virus modified by the presence of the polynucleotide construct in its genome, at a site which does not affect virus viability.

As noted above, the gene is inserted into a region (insertion region), in the virus which does not affect virus viability of the resultant recombinant virus. The skilled artisan can readily identify such regions in a virus by, for example, randomly testing segments of virus DNA for regions that allow recombinant formation without seriously affecting virus viability of the recombinant. One region that can readily be used and is present in many viruses is the thymidine kinase gene.

Techniques for preparing replication-defective adenoviruses are well known in the art, as exemplified by Ghosh-Choudhury and Graham (1987) *Biochem. Biophys. Res. Comm.* 147:964-973; Ghosh-Choudhury et al. (1987) *EMBO J.* 6:1733-1739; McGrory et al. (1988) *Virology* 163:614-617. It is also well known that various cell lines may be used to propagate recombinant adenoviruses, so long as they complement any replication defect which may be present. One example is the human 293 cell line, but any other cell line that is permissive for replication. For example, for viral constructs which, by virtue of insertion of an *hUPII* transcriptional regulatory polynucleotide, E1A and E1B are not expressed, a cell line which

expresses E1A and E1B is employed. Further, the cells can be propagated either on plastic dishes or in suspension culture, in order to obtain virus stocks.

Preparation of replication-competent adenoviral vectors is discussed in a separate section.

5 Recombinant retroviruses which are constructed to carry or express an *hUPII* transcriptional regulatory polynucleotide can be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see *RNA Tumor Viruses*, Second Edition, Cold Spring Harbor Laboratory, 1985). Such retroviruses may be readily utilized in order
10 to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (e.g., Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS* 82:488, 1985). In addition, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, retroviral
15 LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

 Insertion of an IRES into a vector is accomplished by methods and techniques that are known in the art and described herein *supra*, including but not limited to,
20 restriction enzyme digestion, ligation, and PCR. A DNA copy of an IRES can be obtained by chemical synthesis, or by making a cDNA copy of, for example, a picornavirus IRES. See, for example, Duke et al. (1995) *J. Virool.* 66(3):1602-9) for a description of the EMCV IRES and Huez et al. (1998), *Mol. Cell. Biol.* 18(11):6178-90) for a description of the VEGF IRES. The internal translation initiation sequence
25 is inserted into a vector genome at a site such that it lies upstream of a 5'-distal coding region in a multicistronic mRNA. For example, in a preferred embodiment of an adenovirus vector in which production of a bicistronic E1A-E1B mRNA is under the control of a target cell-specific TRE, the E1B promoter is deleted or inactivated,

and an IRES sequence is placed between E1A and E1B. In other embodiments disclosed herein, the 19-kDa region of E1B is deleted. IRES sequences of cardioviruses and certain aphthoviruses contain an AUG codon at the 3' end of the IRES that serves as both a ribosome entry site and as a translation initiation site.

5 Accordingly, this type of IRES is introduced into a vector so as to replace the translation initiation codon of the protein whose translation it regulates. However, in an IRES of the entero/rhinovirus class, the AUG at the 3' end of the IRES is used for ribosome entry only, and translation is initiated at the next downstream AUG codon. Accordingly, if an entero/rhinovirus IRES is used in a vector for translational
10 regulation of a downstream coding region, the AUG (or other translation initiation codon) of the downstream gene is retained in the vector construct.

A major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly regarding the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines ("packaging cells") which
15 produce only replication-defective retroviruses has increased the utility of retroviruses for gene delivery, and defective retroviruses are well characterized for gene delivery purposes. Miller et al. (1990) *Blood* 76:271. Recombinant retroviruses can be constructed in which part of the retroviral coding sequence (gag, pol, env) has been replaced by an *hUPII* transcriptional regulatory polynucleotide, rendering the
20 retrovirus replication defective. The replication-defective virus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* can be found in Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds) Greene Publishing Associates (1989) and periodic
25 updates, and other standard laboratory manuals.

Packaging cell lines suitable for use with the above-described vector constructs may be readily prepared (see WO 92/05266), and utilized to create

producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles, given the disclosure provided herein.

Introduction into host cells and/or target cells of non-viral vehicles comprising an hUPII transcriptional regulatory polynucleotide

Non-viral vehicles comprising an *hUPII* transcriptional regulatory polynucleotide may be introduced into host cells and/or target cells by any method known in the art, such as transfection by the calcium phosphate coprecipitation technique; electroporation; electropermeabilization; liposome-mediated transfection; ballistic transfection; biolistic processes including microparticle bombardment, jet injection, and needle and syringe injection; or by microinjection. Numerous methods of transfection are known to the skilled worker in the field. A number of these methods can be carried out both *ex vivo* and *in vivo*. Biolistic gene transfer, including jet injection, microparticle bombardment and needle and syringe injection, can be carried out by art-known methods. For a review, see Furth (1997) *Mol. Biotechnol.* 7:139-143. *In vivo* electropermeabilization can be performed as described. Rols et al. (1998) *Nature Biotech.* 16:168-1171. Successful transfection is generally recognized when any indication of the operation of this vector occurs in the host cell. Transformation is achieved using standard techniques appropriate to the particular host cells used. Naked DNA can be introduced by direct injection. Polynucleotides can also be introduced using various implantable devices such as those described in U.S. Patent No. 5,501,662; and Koole et al. (1998) *Nature Biotech.* 16:172-176.

Introduction into host cells and/or target cells of viral vehicles comprising an hUPII transcriptional regulatory polynucleotide

Viral delivery vehicles can be introduced into cells by infection. Alternatively, viral vehicles can be incorporated into any of the non-viral delivery vehicles described above for delivery into cells. For example, viral vectors can be

mixed with cationic lipids (Hodgson and Solaiman (1996) *Nature Biotechnol.* 14:339-342); or lamellar liposomes (Wilson et al. (1977) *Proc. Natl. Acad. Sci. USA* 74:3471; Faller et al. (1984) *J. Virol.* 49:269).

For *in vivo* delivery, the delivery vehicle(s) can be introduced into a patient by any of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systematically, e.g. by intravenous injection, and specific transduction of the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments initial delivery of the recombinant gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Pat. No. 5,328,470) or by stereotactic injection (e.g. Chen et al. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:3054-3057). Moreover, the pharmaceutical preparation can consist essentially of the gene delivery system in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery system can be produced intact from recombinant cells, e.g. retroviral packages, the pharmaceutical preparation can comprise one or more cells which produce the gene delivery system. In the case of the latter, methods of introducing the viral packaging cells may be provided by, for example, rechargeable or biodegradable devices. Various slow release polymeric devices have been developed and tested *in vivo* in recent years for the controlled delivery of drugs, including proteinacious biopharmaceuticals, and can be adapted for release of viral particles through the manipulation of the polymer composition and form. A variety of biocompatible polymers (including hydrogels), including both biodegradable and non-degradable polymers, can be used to form an implant or the sustained release of an the viral particles by cells implanted at a particular target site. Such embodiments of the

present invention can be used for the delivery of an exogenously purified virus, which has been incorporated in the polymeric device, or for the delivery of viral particles produced by a cell encapsulated in the polymeric device.

By choice of monomer composition or polymerization technique, the amount of water, porosity and consequent permeability characteristics can be controlled. The selection of the shape, size, polymer, and method for implantation can be determined on an individual basis according to the disorder to be treated and the individual patient response. The generation of such implants is generally known in the art. See, for example, Concise Encyclopedia of Medical & Dental Materials, ed. by David Williams (MIT Press: Cambridge, MA, 1990); and the Sabel et al. U.S. Pat. No. 4,883,666. In another type of implant, a source of cells producing the recombinant virus is encapsulated in implantable hollow fibers. Such fibers can be pre-spun and subsequently loaded with the viral source (Aebischer et al. U.S. Pat. No. 4,892,538; Aebischer et al. U.S. Pat. No. 5,106,627; Hoffman et al. (1990) Expt. Neurobiol. 110:39-44; Jaeger et al. (1990) Prog. Brain Res. 82:41-46; and Aebischer et al. (1991) J. Biomech. Eng 113: 178-183), or can be co-extruded with a polymer which acts to form a polymeric coat about the viral packaging cells (Lim U.S. Pat. No. 4,391,909; Sefton U.S. Pat. No. 4,353,888; Sugarmori et al. (1989) Trans. Am. Artif. Intern. Organs 35:791-799; Sefton et al. (1987) Biotechnol. Bioeng. 29:1135-1143; and Aebischer et al. (1991) Biomaterials 12:50-55. Again, manipulation of the polymer can be carried out to provide for optimal release of viral particles.

Host cells and target cells comprising an hUPII transcriptional regulatory polynucleotide

The invention further provides host cells and target cells transfected or transformed with (i.e., comprising) the above-described *hUPII* transcriptional regulatory sequences and/or *hUPII*-TRE(s), above-described expression or cloning vectors of this invention, or above-described delivery vehicles comprising *hUPII* transcriptional regulatory sequences and/or *hUPII*-TRE(s). These cells are cultured

in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences.

The cells which are suitable for use in the methods of the present invention with respect to expression, transcriptional control, or for purposes of cloning and propagating an *hUPII* transcriptional regulatory polynucleotide can be prokaryotic or eukaryotic.

Host systems are known in the art and need not be described in detail herein. Prokaryotic hosts include bacterial cells, for example *E. coli*, *B. subtilis*, and mycobacteria. Among eukaryotic hosts are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian cells. Examples of fungi (including yeast) host cells are *S. cerevisiae*, *Kluyveromyces lactis* (*K. lactis*), species of *Candida* including *C. albicans* and *C. glabrata*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* (*S. pombe*), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are COS cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells. *Xenopus laevis* oocytes, or other cells of amphibian origin, may also be used.

For the delivery vehicles described above, any eukaryotic cells, preferably mammalian cells can be used. Even more preferably, the cells are urothelial cells, such as bladder urothelial cells. The cells employed may be those derived from the bladder. Such cells include, but are not limited to, the cell lines SW780 (bladder cell carcinoma; available from the American Type Culture Collection under ATCC CRL-2169), UM-UC-3 (bladder cell carcinoma; available from the American Type Culture Collection under ATCC CRL-1749). Alternatively, the cells need not be derived from the bladder as long as the *hUPII*-TRE function is sufficiently preserved. This may be achieved, for example, by co-transfecting the cell with a gene encoding a product necessary for the function of the TRE of the urothelial cell-specific gene. For example, if an *hUPII*-TRE is inducible by a hormone, it may be necessary to co-

transfect into the cells a construct which encodes and allows expression of a gene encoding the corresponding hormone receptor.

The host cells of this invention can be used, *inter alia*, as repositories of *hUPII* polynucleotides and/or vehicles for production of *hUPII* polynucleotides and/or polypeptides which are encoded by an operably linked polynucleotide.

Compositions containing cells into which have been introduced vectors comprising an *hUPII*-TRE operably linked to a heterologous polynucleotide are encompassed by this invention. When these compositions are to be used pharmaceutically, they are combined with a pharmaceutically acceptable excipient. Accordingly, the invention also provides compositions of these cells, including compositions comprising these cells and a pharmaceutical excipient, as well pharmaceutical compositions comprising these cells. Pharmaceutical excipients are well known in the art and need not be described in detail herein. See, for example, *Remington: The Science and Practice of Pharmacy* (19th edition, 1995), Gennaro, ed.

An example of a composition provided by the invention is a composition for expressing an anti-proliferation construct in a urothelial cell. The composition comprises, in addition to a pharmaceutically acceptable excipient, an *hUPII*-TRE a polynucleotide of claim 1 operably linked to a coding sequence for an anti-proliferation molecule, such as a toxin, an antigen, a lymphokine, a viral sequence, and/or an antisense sequence.

Methods using the *hUPII* transcriptional regulatory polynucleotides of the invention

The above-described *hUPII* transcriptional regulatory sequences can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the *hUPII* transcriptional regulatory sequences described above.

As described above, an *hUPII* transcriptional regulatory sequence can be operably linked to a heterologous polynucleotide. Such an *hUPII*-TRE is useful for

selectively increasing transcription and/or translation of an operably linked heterologous polynucleotide in cells which allow an *hUPII* transcriptional regulatory sequence to function. Accordingly, the invention includes methods for increasing transcription of a polynucleotide sequence in a cell, generally involving introducing a construct comprising an *hUPII* transcriptional regulatory sequence operably linked to the polynucleotide into a cell in which the *hUPII* transcriptional regulatory sequence is functional, such as a urothelial cell. The polynucleotide sequence which is operably linked to the *hUPII* transcriptional regulatory sequence may be any sequence, including, but not limited to, a heterologous coding sequence such as a reporter gene, a toxin, a lymphokine.

In one embodiment, methods are provided for introducing a construct comprising an *hUPII*-TRE operably linked to a reporter gene into cells which allow an *hUPII* transcriptional regulatory sequence to function, i.e., a cell in which an *hUPII* transcriptional regulatory sequence, when operably linked to a promoter and a reporter gene, increases expression of the reporter gene. Examples include cells as shown in Example 2. Such cells are useful for screening compounds for therapeutic effect against bladder cancer. Methods for screening candidate compounds are described below.

In another embodiment, methods are provided for conferring selective cytotoxicity in cells in which an *hUPII*-TRE is functional, comprising contacting the cells with a delivery vehicle described herein, wherein the vehicle enters the cell such that transcription of the polynucleotide is operably linked to an *hUPII*-TRE which contributes to cytotoxicity. Preferably, the vehicle is a viral vector. Preferably, the viral vector is adenovirus. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ³H-thymidine incorporation, and/or lysis.

In another embodiment, methods are provided for the selective transcription and/or expression of a heterologous polynucleotide in cells which the function of an *hUPII* transcriptional regulatory sequence is sufficiently preserved. By "sufficiently

preserved", it is intended that transcription due to the presence of the transcriptional regulatory sequence is increased above basal levels (i.e., promoter alone; lacking enhancer) in the target cell by at least about 2-fold, preferably at least about 5-fold, preferably at least about 10-fold, more preferably at least about 20-fold, more preferably at least about 50-fold, more preferably at least about 100-fold, more preferably at least about 200-fold, even more preferably at least about 400- to about 500-fold, even more preferably at least about 1000-fold.

In particular, methods are provided for selective transcription and/or expression of a heterologous polynucleotide in cells which do not normally express the heterologous polynucleotide, or express the heterologous polynucleotide at undetectable levels. Expression of the heterologous polynucleotide by such cells can be detected in a variety of ways, including but not limited to, fluorescence-activated cell sorting (FACS) using one or more antibodies specific for a protein expressed on a cell surface (in situations in which the heterologous polynucleotide expresses a product which is expressed on the cell surface), enzyme-linked immunoassay (ELISA) of cell supernatants (for a secreted product of a heterologous polynucleotide), using an antibody specific for the secreted product.

Accordingly, the invention provides methods for increasing transcription of an operably linked polynucleotide sequence in a cell comprising introducing a construct comprising an *hUPII* transcriptional regulatory sequence operably linked to said polynucleotide into a cell in which the *hUPII* transcriptional regulatory sequence is functional. Such cells have been described above, as have *hUPII* transcriptional regulatory sequences (i.e., polynucleotide sequences having transcriptional regulatory activity).

The invention also provides (a) methods of detecting *hUPII* transcriptional regulatory sequences and (b) methods of amplifying *hUPII* transcriptional regulatory sequences. Detection methods generally entail contacting a suitable probe (described herein) with a polynucleotide in a sample under conditions that permit formation of a

duplex and detecting the duplex, if any. An amplification (which may or may not be used for detection), such as PCR, generally involves using a suitable primer under conditions such that a target sequence is amplified. Such manipulations are well known in the art.

5 The invention also provides methods for expressing a polynucleotide coding sequence in a urothelial cell, said method comprising (a) introducing a vector comprising said coding sequence operably linked to a polynucleotide according to claim 1 into the urothelial cells; and expressing the coding sequence.

10 ***Screening methods utilizing an hUPII-TRE***

The present invention provides methods for screening compounds which affect transcriptional regulatory function of an *hUPII*-TRE. Such compounds may be useful for treatment of bladder cancer. These screening methods employ an expression construct which comprises an *hUPII* transcriptional regulatory element (*hUPII*-TRE) (comprising any of the *hUPII* transcriptional regulatory sequences described herein) and a reporter gene under the transcriptional control of an *hUPII*-TRE whose expression product provides a detectable signal. The method comprises the steps of:

- 15 a) combining cells with a candidate compound in the presence of an appropriate inducing agent for a sufficient time for detectable expression of the reporter gene; and
- 20 b) detecting the level of expression of the reporter gene as compared to the level of expression in the absence of the candidate compound.

Accordingly, the invention provides methods for screening for compounds which alter expression of a urothelial cell-specific gene, said method employing cells containing an expression construct, said expression construct comprising an *hUPII* TRE and a marker gene whose expression produce provides a detectable signal, wherein said marker gene is under the transcriptional control of the *hUPII* TRE, and the cell allows function of the *hUPII* TRE, then method comprising (a) combining

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the cells with a candidate compound and incubating the cells for a sufficient time for detectable expression of the marker gene and (b) detecting the level of expression of the marker gene as compared to the level of expression in the absence of the compound. An alteration of expression in the presence of the compound indicates that the compound alters urothelial cell-specific expression.

The screening methods involve introducing an expression construct comprising an *hUPII*-TRE operably linked to a reporter gene into cells which allow an *hUPII*-TRE to function. An *hUPII*-TRE can be operably linked to a reporter gene and inserted into a variety of vectors. Host cells are then transfected or transformed with vectors containing an *hUPII*-TRE linked to a reporter gene and cultured in conventional nutrient media modified as appropriate for selecting transformants, for example.

Cell-based screening assays of the present invention can be designed, *e.g.*, by constructing cell lines in which the expression of a reporter protein, *i.e.*, an easily assayable protein, such as β -galactosidase, chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP) or luciferase, is dependent on the function of an *hUPII*-TRE. For example, a DNA construct comprising an *hUPII*-TRE may be operably linked to a gene encoding luciferase using methods well known in the art. The resulting DNA construct comprising the luciferase-encoding DNA is stably or transiently transfected into a host cell. The cell is exposed to a test compound and an appropriate inducing agent if necessary, such as a hormone, and, after a time sufficient to effect luciferase expression, the cells are assayed for the production of luciferase by standard enzyme assays.

Reporter genes which may be employed are known to those skilled in the art and include, but are not limited to, luciferase; aequorian (*i.e.*, green fluorescent protein from *Aequorea victoria*); β -galactosidase, chloramphenicol acetyl transferase; immunologically detectable protein "tags" such as human growth hormone; and the like. See, for example, Current Protocols in Molecular Biology (F.M. Ausubel et al.,

eds., 1987) and periodic updates. Any assay which detects a product of the reporter gene, either by directly detecting the protein encoded by the reporter gene or by detecting an enzymatic product of a reporter gene-encoded enzyme, is suitable for use in the present invention. Assays include colorimetric, fluorimetric, or luminescent assays or even, in the case of protein tags, radioimmunoassays or other immunological assays.

A recombinant polynucleotide comprising an *hUPII*-TRE or active fragment thereof, as well as those which may comprise other *hUPII* transcriptional regulatory elements described herein, may be prepared by any technique to those of skill in the art using the sequence information provided herein.

A construct may be incorporated into a suitable vector for the purposes of propagation or expression. Such vectors include prokaryotic plasmids, eukaryotic plasmids and viral vectors, and the choice of vector depends upon the design of the screening assay, the cell types involved and other factors. Expression constructs comprising an *hUPII*-TRE include plasmid and viral vectors, particularly adenovirus vectors, as described herein.

For preparing an expression construct comprising an *hUPII*-TRE operably linked to a reporter gene for use in the screening methods of the present invention, a polynucleotide comprising an *hUPII*-TRE operably linked to a reporter gene can be inserted into a suitable vector, and the vector in turn can be introduced into a suitable host cell for replication and amplification. Polynucleotides may be inserted into host cells by any means known in the art. Cells are transformed by introducing an exogenous polynucleotide by direct uptake, endocytosis, transfection, f-mating or electroporation. Once introduced, the exogenous polynucleotide can be maintained within the cell as a non-integrated vector (such as a plasmid) or integrated into the host cell genome. The polynucleotide so amplified can be isolated from the host cell by methods well known within the art. See, e.g., Sambrook et al. (1989).

5 The cells which are suitable for use in the methods of the present invention with respect to screening of compounds for possible therapeutic use in treatment of bladder cancer are any eukaryotic cells, preferably mammalian cells, which allow an *hUPII*-TRE to function. Preferably, the cells are urothelial cells, such as those described in the Examples.

10 An inducing agent can be any compound which is added to the growth environment of the cell and which, upon contact with and/or entry into the cell, results in the transcriptional activation of an *hUPII*-TRE. For the purposes of the present invention, an "appropriate inducing agent" is one which specifically induces the expression of an *hUPII*-TRE which is operably linked to a reporter gene. An example of an inducing agent used is a hormone.

15 Once an *hUPII*-TRE-reporter gene construct has been introduced into the host cell and stable cell lines are made, the cells may be cultured in a suitable growth medium, then exposed to an agent whose ability to modulate the activity of the *hUPII*-TRE is to be tested.

20 Stable cell lines comprising an expression construct which comprises an *hUPII*-TRE driving expression of a reporter gene can be generated for use in the screening methods, as described above. Alternatively, appropriate cells can be transiently transfected with the expression constructs, the cells cultured in a suitable growth medium, then exposed to an agent whose ability to modulate the activity of the *hUPII*-TRE is to be tested. Methods for transient transfection are known in the art.

25 The reporter gene used can encode luciferase activity, and an assay system can be chosen such that the product of the luciferase activity is luminescent. Luminescence may be determined in accordance with conventional commercial kits, e.g. enhanced luciferase assay kit (Analytical Luminescence Laboratory, MI). The cells may be distributed in multiwell plates which can be accommodated by a luminometer. A known number of cells is introduced into each one of the wells in an

appropriate medium, the candidate compound added, and the culture maintained for at least 12 hours, more usually at least about 24, and not more than about 60 hours, particularly about 48 hours. The culture is then lysed in an appropriate buffer, using a non-ionic detergent, e.g. 1% triton X-100. The cells are then promptly assayed.

5 After a suitable time, cells are tested for amount of reporter gene product. A “suitable time” in this assay means an amount of time sufficient for the agent to be tested to effect a change in the levels of reporter gene product such that a difference from the control can be measured. This amount of time may depend on the stability of the reporter gene messenger RNA or protein, on how readily the agent enters the
10 cell, on how stable the agent is once it enters the cell, and/or on other factors. In general, a suitable time must be determined empirically and this is well within the skill of one of ordinary skill in the art. A decrease or increase in the level of reporter gene product of from at least about 25% to about 40%, more preferably from at least about 40% to about 70%, and most preferably from about 70% to about 100% is
15 indicative of an agent that modulates the activity of an *hUPII*-TRE.

Assay methods generally require comparison to a control sample to which no agent is added. Modulation of *hUPII* expression is said to be effected by a test agent if such an effect does not occur in the absence of the test agent.

In another embodiment, the above-described *hUPII*-TRE-reporter gene
20 plasmid constructs may also be introduced into the host cells for transient expression of the reporter gene. In this assay system, the compound to be tested may be added before or simultaneously with introduction of the plasmid into the cells. To correct for differences in transfection efficiency, the cells can be co-transfected with a reference plasmid encoding, for example, β -galactosidase. The cells are then cultured
25 for a time, after which the level of reporter gene product is measured and, if appropriate, the product encoded by the plasmid serving as a transfection efficiency control is also measured. The ability of the agent to modulate the activity of an

hUPII-TRE is measured as a difference in the amount of reporter gene product relative to control cell culture to which no test compound was added.

In a further embodiment of the present invention, an *hUPII*-TRE operably linked to a reporter gene may be incorporated into a viral vector for packaging into a viral particle. The virus may be any known in the art which can infect eukaryotic cells. Preferably, adenovirus is used. An *hUPII*-TRE-reporter gene may be incorporated into an adenoviral vector at a variety of sites. Preferably one or more genes essential for adenovirus replication are replaced with an *hUPII*-TRE-reporter gene construct. For example, the regions known as E1A and E1B can be replaced with a fragment of DNA containing an *hUPII*-TRE operably linked to a reporter gene. The resulting adenovirus construct can be propagated by passage through a cell line that provides the E1A and E1B gene products, *e.g.* 293 cells, by methods known in the art. In this assay system, the adenovirus construct containing an *hUPII*-TRE operably linked to a reporter gene can be used to infect an appropriate cell line such as those described above. An agent whose ability to modulate the activity of an *hUPII*-TRE can be added either simultaneously with the adenoviral construct or after a suitable time. A "suitable time" in this assay system means an amount of time sufficient to allow entry of the viral particle into the cell, subsequent uncoating of the viral particle, and transport into the nucleus. This amount of time may be from about one to about five hours. After culturing the cells in an appropriate growth medium, the levels of reporter gene product are measured and compared to levels in recombinant host cell cultures to which no agent has been added.

Compounds can be tested singly or in combination with one another. Thus, screening assays provide a method for identifying an "agent," which can be used to modulate *hUPII* expression in a cell *in vitro* or in a patient. An "effective agent" is one that modulates *hUPII* expression.

As used herein, the term "modulate" means that the effective agent can increase or decrease the level of expression of a gene under transcriptional control of

an *hUPII*-TRE or an active fragment thereof. Modulation can occur as a result of an effect at any point in signal transduction from the membrane of the cell to the nucleus. The ways that an effective agent can act to modulate the expression of *hUPII* include, but are not limited to 1) modifying binding of a transcription factor to an *hUPII*-TRE; 2) modifying the interaction between two transcription factors necessary for *hUPII* expression; 3) altering the ability of a transcription factor necessary for *hUPII* expression to enter the nucleus; 4) inhibiting the activation of a transcription factor involved in *hUPII* gene transcription; 5) modifying a cell-surface receptor which normally interacts with a ligand and whose binding of the ligand results in *hUPII* expression; 6) inhibiting the inactivation of a component of the signal transduction cascade that leads to *hUPII* expression; and 7) enhancing the activation of a transcription factor involved in *hUPII* gene transcription.

Adenoviral vectors comprising a urothelial cell-specific TRE

The present invention also provides replication-competent adenoviral vector constructs which comprise a gene, preferably an adenovirus gene, under transcriptional control of a urothelial cell-specific TRE. Preferably, the adenovirus gene contributes to cytotoxicity (whether direct and/or indirect), more preferably is one that contributes to or causes cell death, even more preferably is essential for adenoviral replication. Examples of a gene that contributes to cytotoxicity include, but are not limited to, adenovirus death protein (ADP). When the adenovirus vector(s) is selectively (i.e., preferentially) replication competent for propagation in target cells, i.e., urothelial cells, these cells will be preferentially killed upon adenoviral proliferation. Once the target cells are destroyed due to selective cytotoxic and/or cytolytic replication, the adenovirus vector replication is significantly reduced, thus lessening the probability of runaway infection and undesirable bystander effects. *In vitro* cultures may be retained to monitor the mixture (such as, for example, a biopsy or other appropriate biological sample) for occurrence (i.e., presence) and/or recurrence of the target cell, e.g., a neoplastic cell

or other undesired cell. To further ensure cytotoxicity, one or more transgenes having a cytotoxic effect may also be present and under selective transcriptional control. In this embodiment, one may provide higher confidence that the target cells will be destroyed. Additionally, or alternatively, an adenovirus gene that contributes to cytotoxicity and/or cell death (such as ADP) may be included in the adenoviral vector, either free of, or under, selective transcriptional control.

Significantly, we have observed that such constructs are capable of selectively replicating in urothelial cells as opposed to smooth muscle cells, which adjoin urothelial cells in the bladder.

Urothelial cell-specific TREs

Any urothelial cell-specific TRE may be used in the adenoviral vectors of the invention. Preferred urothelial cell-specific TREs include TREs derived from the uroplakins UPIa, UPIb, UPII, and UPIII, as well as urohingin. A uroplakin TRE may be from any species, depending on the intended use of the adenovirus, as well as the requisite functionality is exhibited in the target or host cell.

For example, urothelial-specific TREs derived from the *hUPII* gene were described above. Accordingly, in some embodiments, an adenovirus vector of the invention comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises the 2.2 kb sequence from the 5' flanking region of *hUPII* gene, as shown in FIG. 1. In other embodiments, an adenovirus vector of the invention comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises a 1.8 kb sequence from the 5' flanking region of *hUPII* gene, as shown in nucleotides 430 to 2239 of FIG. 1. In other embodiments, the urothelial cell-specific TRE comprises a functional portion of the 2.2 kb sequence depicted in FIG. 1, such as a fragment of about 2000 bp or less, about 1500 bp or less, about 1000 bp or less, about 600 bp less, or at least 200 bp which includes the 200 bp fragment of the *hUPII* 5'-flanking region

as described above. Other embodiments of *hUPII* transcriptional regulatory sequences suitable for the adenovirus vectors are described above and are included in the invention.

5 A 3.6 kb 5'-flanking sequence located from the mouse UPII (mUPII) gene which confers urothelial cell-specific transcription on heterologous genes is one urothelial cell-specific TRE useful in vectors of the instant invention (FIG. 2, SEQ ID NO:2). Smaller TREs (*i.e.*, 3500 bp or less, more preferably less than about 2000 bp, 1500 bp, or 1000 bp) are preferred. Smaller TREs derived from the mUPII 3.6 kb fragment are one group of preferred urothelial cell-specific TREs. In particular, 10 Inventors have identified an approximately 600 bp fragment from the 5' flanking DNA of the mUPII gene, which contains 540 bp of 5' untranslated region (UTR) of the mUPII gene, that confers urothelial cell-specific expression on heterologous genes.

Accordingly, in some embodiments, an adenovirus vector of the invention 15 comprises an adenovirus gene, preferably an adenoviral gene essential for replication, under transcriptional control of a urothelial cell-specific TRE which comprises the 3.6 kb sequence from the 5' flanking region of mouse UPII gene, as shown in FIG. 2. In other embodiments, the urothelial cell-specific TRE comprises a functional portion of the 3.6 kb sequence depicted in FIG. 2, such as a fragment of about 3500 bp or less, about 2000 bp or less, about 1500 bp or less, or about 1000 bp or less which 20 includes the 540 bp fragment of 5' UTR. The urothelial cell-specific TRE may also be a sequence which is substantially identical to the 3.6 kb mUPII 5'-flanking region or any of the described fragments thereof.

25 A urothelial cell-specific TRE can also comprise multimers. For example, a urothelial cell-specific TRE can comprise a tandem series of at least two, at least three, at least four, or at least five urothelial cell-specific TREs. These multimers may also contain heterologous promoter and/or enhancer sequences.

Optionally, a transcriptional terminator or transcriptional "silencer" can be placed upstream of the urothelial cell-specific TRE, thus preventing unwanted read-through transcription of the coding segment under transcriptional control of the urothelial cell-specific TRE. Also, optionally, the endogenous promoter of the coding segment to be placed under transcriptional control of the urothelial cell-specific TRE can be deleted.

A urothelial cell-specific TRE may or may not lack a silencer. The presence of a silencer (i.e., a negative regulatory element) may assist in shutting off transcription (and thus replication) in non-permissive cells (i.e., a non-urothelial cell). Thus, presence of a silencer may confer enhanced urothelial cell-specific replication by more effectively preventing adenoviral vector replication in non-target cells. Alternatively, lack of a silencer may assist in effecting replication in target cells, thus conferring enhanced urothelial cell-specific replication due to more effective replication in target cells.

It is also understood that other, heterologous, TREs may be included in the adenoviral vectors of this invention, and that these additional TREs may or may not be operably linked to the same gene(s) as the urothelial cell-specific TRE. For example a TRE (such as a cell type-specific or cell status-specific TRE) may be juxtaposed to a urothelial cell-specific TRE. "Juxtaposed" means a urothelial cell-specific TRE and a second TRE transcriptionally control the same gene. For these embodiments, the urothelial cell-specific TRE and the second TRE may be in any of a number of configurations, including, but not limited to, (a) next to each other (i.e., abutting); (b) both 5' to the gene that is transcriptionally controlled (i.e., may have intervening sequences between them); (c) one TRE 5' and the other TRE 3' to the gene.

As is readily appreciated by one skilled in the art, a urothelial cell-specific TRE is a polynucleotide sequence, and, as such, can exhibit function over a variety of sequence permutations. Methods of nucleotide substitution, addition, and deletion

are known in the art, and readily available functional assays (such as the CAT or luciferase reporter gene assay) allow one of ordinary skill to determine whether a sequence variant exhibits requisite urothelial cell-specific transcription function. Hence, the invention also includes functionally-preserved variants of the nucleic acid sequences disclosed herein, which include nucleic acid substitutions, additions, and/or deletions. The variants of the sequences disclosed herein may be 80%, 85%, 90%, 95%, 98%, 99% or more identical, as measured by, for example, ALIGN Plus (Scientific and Educational Software, Pennsylvania), preferably using default parameters, which are as follows: mismatch = 2; open gap = 0; extend gap = 2 to any of the urothelial cell-specific TRE sequences disclosed herein. Variants of urothelial cell-specific TRE sequences may also hybridize at high stringency to any of the urothelial cell-specific TRE sequences disclosed herein. While not wishing to be bound by a single theory, the inventors note that it is possible that certain modifications will result in modulated resultant expression levels, including enhanced expression levels. Achievement of modulated resultant expression levels, preferably enhanced expression levels, may be especially desirable in the case of certain, more aggressive forms of cancer, or when a more rapid and/or aggressive pattern of cell killing is warranted (due to an immunocompromised condition of the individual, for example).

As an example of how urothelial cell-specific TRE activity can be determined, a polynucleotide sequence or set of such sequences can be generated using methods known in the art, such as chemical synthesis, site-directed mutagenesis, PCR, and/or recombinant methods. The sequence(s) to be tested is inserted into a vector containing an appropriate reporter gene, including, but not limited to, chloramphenicol acetyl transferase (CAT), β -galactosidase (encoded by the lacZ gene), luciferase (encoded by the luc gene), a green fluorescent protein, alkaline phosphatase, and horse radish peroxidase. Such vectors and assays are readily available, from, inter alia, commercial sources. Plasmids thus constructed are

transfected into a suitable host cell to test for expression of the reporter gene as controlled by the putative urothelial cell-specific TRE using transfection methods known in the art, such as calcium phosphate precipitation, electroporation, liposomes (lipofection) and DEAE dextran. Suitable host cells include any urothelial cell type, including but not limited to, KU-1, MYP3 (a non-tumorigenic rat urothelial cell line), 804G (rat bladder carcinoma cell line), cultured human urothelial cells (HUC), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. Non-urothelial cells, such as LNCaP, HBL-100, HLF, HLE, 3T3, Hep3B, HuH7, CADO-LC9, and HeLa are used as a control. Results are obtained by measuring the level of expression of the reporter gene using standard assays. Comparison of expression between urothelial cells and control indicates presence or absence of transcriptional activation.

Comparisons between or among various urothelial cell-specific TREs can be assessed by measuring and comparing levels of expression within a single urothelial cell line. It is understood that absolute transcriptional activity of a urothelial cell-specific TRE will depend on several factors, such as the nature of the target cell, delivery mode and form of the urothelial cell-specific TRE, and the coding sequence that is to be selectively transcriptionally activated. To compensate for various plasmid sizes used, activities can be expressed as relative activity per mole of transfected plasmid. Alternatively, the level of transcription (i.e., mRNA) can be measured using standard Northern analysis and hybridization techniques. Levels of transfection (i.e., transfection efficiencies) are measured by co-transfecting a plasmid encoding a different reporter gene under control of a different TRE, such as the CMV immediate early promoter. This analysis can also indicate negative regulatory regions, i.e., silencers.

Alternatively a putative urothelial cell-specific TRE can be assessed for its ability to confer adenoviral replication preference for cells that allow a urothelial cell-specific TRE to function. For this assay, constructs containing an adenovirus gene essential to replication operatively linked to a putative urothelial cell-specific TRE

are transfected into urothelial cells. Viral replication in those cells is compared, for example, to viral replication by wild type adenovirus in those cells and/or viral replication by the construct in non-urothelial cells. A more detailed description of this kind of assay is in Example 3.

5 It is understood that, to make and use the instant adenoviral vectors, it is not necessary to use urothelial cell-specific TREs having maximum activity, or having minimum size. The requisite degree of activity is determined, inter alia, by the anticipated use and desired result. For example, if an adenoviral vector of the invention is used to monitor cells for urothelial cell-specific TRE activity, it is possible that less than a maximal degree of responsiveness by a urothelial cell-specific TRE will suffice to qualitatively indicate the presence of such cells. Similarly, if used for treatment or palliation of a disease state, less-than-maximal responsiveness may be sufficient for the desired result, if, for example, the urothelial cells, such as transitional cell carcinoma cells, are not especially virulent and/or the extent of disease is relatively confined.

10 Various replication-competent adenovirus vectors can be made according to the present invention in which a single or multiple adenovirus gene(s) are under control of a urothelial cell-specific TRE.

15 For example, a urothelial cell-specific TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to a gene which is a replication gene, e.g. an early gene such as E1A or E1B or a late gene such as L1, L2, L3, L4, or L5. Optionally, the endogenous adenovirus promoter for the replication gene is deleted, placing the gene under sole transcriptional control of a urothelial cell-specific TRE. Alternatively, a urothelial cell-specific TRE can be placed immediately upstream of and operably linked to an ADP (adenovirus death protein) gene.

20 In some embodiments, a urothelial cell-specific TRE is used with an adenovirus gene that is essential for propagation, so that replication competence is

preferentially achievable in a target cell that allow a urothelial cell-specific TRE to function. Preferably, the gene is an early gene, such as E1A, E1B, E2, or E4. (E3 is not essential for viral replication.) More preferably, the early gene under urothelial cell-specific TRE control is E1A and/or E1B. More than one early gene can be placed under control of a urothelial cell-specific TRE. Example 3 provides a more detailed description of adenoviral constructs in which E1A is under transcriptional control of urothelial cell-specific TREs.

In one embodiment, an early gene such as E1A or E1B gene is under control of a urothelial cell-specific TRE. In one embodiment, E1A and E1B are under control of one or more urothelial cell-specific TREs by making the following construct. A fragment containing the coding region of E1A through the E1B promoter is excised from the Ad genome and reinserted in opposite orientation. In this configuration, the E1A and E1B promoters are next to each other, followed by E1A in opposite orientation (so that neither the E1A or E1B promoters are operatively linked to E1A), followed by E1B in opposite orientation with respect to E1A. A urothelial cell-specific TRE(s) can be inserted between E1A and E1B coding regions, (which are in opposite orientation), so that these regions are under control of the TRE(s). Appropriate promoter sequences are inserted proximal to the E1A and E1B region as shown in FIG. 3. Thus, an urothelial cell-specific TRE may drive both E1A and E1B. Such a configuration may prevent, for example, possible loop-out events that may occur if two urothelial cell-specific TREs were inserted in intact (native) Ad genome, one each 5' of the coding regions of E1A and E1B. By introducing a polycloning site between E1A and E1B, other types of TREs can be inserted, such as a carcinogen embryonic antigen TRE (CEA-TRE); a mucin TRE (MU-TRE); or other cell-specific regulatory elements, preferably those associated with a disease state, such as neoplasm. Thus, this construct may find general use for cell-specific, temporal, or other means of control of adenovirus genes E1A and E1B,

thereby providing a convenient and powerful way to render adenoviral replication dependent upon a chosen transcriptional parameter.

In some embodiments, the adenovirus death protein (ADP), encoded within the E3 region, is maintained (i.e. contained) in the adenovirus vector. The ADP gene, under control of the major late promoter (MLP), appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement.

Accordingly, the invention provides an adenoviral vector that includes a polynucleotide sequence encoding an ADP. A DNA sequence encoding an ADP and the amino acid sequence of an ADP are depicted in SEQ ID NO:___ and SEQ ID NO:___ respectively (Figure 12). Briefly, an ADP coding sequence is obtained preferably from Ad2 (since this is the strain in which ADP has been more fully characterized) using techniques known in the art, such as PCR. Preferably, the Y leader (which is an important sequence for correct expression of late genes) is also obtained and ligated to the ADP coding sequence. The ADP coding sequence (with or without the Y leader) can then be introduced into the adenoviral genome, for example, in the E3 region (where the ADP coding sequence will be driven by the MLP or the E3 promoter). The ADP coding sequence could also be inserted in other locations of the adenovirus genome, such as the E4 region. The ADP coding sequence could also be operably linked to any of the urothelial-cell specific TREs described herein.

In some embodiments, the invention provides adenoviral vectors which comprise an additional adenovirus gene under transcriptional control of a second urothelial cell-specific TRE. Examples of an additional adenovirus gene under transcriptional control is ADP (discussed above) and genes necessary for replication, such as early genes. For example, an adenoviral vector can be constructed such that a

first urothelial cell-specific TRE regulates transcription of one early gene, such as E1A or E1B, and a second urothelial cell-specific TRE regulates transcription of another early gene. These multiple constructs may be more desirable in that they provide more than one source of cell specificity with respect to replication.

5 Various other replication-competent adenovirus vectors can be made according to the present invention in which, in addition to having an adenovirus gene under control of a urothelial cell-specific TRE, at least one additional gene is placed under control of at least one additional heterologous (non-adenovirus) TRE. This additional TRE(s) can be a cell-, tissue-, and/or cancer-specific TRE. This additional
10 TRE(s) can be another urothelial cell-specific TRE. Optionally, the additional urothelial cell-specific TRE(s) differ from the first. In this way, for example, the possibility of homologous recombination with concomitant loss of intervening sequences can be avoided. The first and additional urothelial cell-specific TREs can, for example, differ in sequence in essential or non-essential regions. For example,
15 the first urothelial cell-specific TRE could comprise a urothelial cell-specific enhancer and a non-urothelial cell-specific promoter; an additional urothelial cell-specific TRE could comprise a non-urothelial cell-specific enhancer and a urothelial cell-specific promoter. Alternatively, the essential portions of the promoter and/or enhancer could be identical in both, with the intervening non-essential regions
20 different. In one embodiment, where one urothelial cell-specific TRE mediates transcription of one gene, and at least one other urothelial cell-specific TRE mediates transcription of another gene, the orientation of the genes is divergent or convergent, rather than tandem. In this way, any recombination between the urothelial cell-specific TREs is unlikely to result to deletion of the intervening sequences.

25 For example, a urothelial cell-specific TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A, and at least one other urothelial cell-specific TRE with a different sequence can be introduced immediately upstream of and operably linked to another early gene

such as E1B. In some embodiments, the adenoviral vector contains two non-identical urothelial TREs that are derived from the same gene or from different genes. In some embodiments, a first TRE is derived from mouse uroplakin II and a second TRE is derived from human uroplakin II.

5 In some embodiments, an adenoviral vector of the invention comprises an adenoviral gene essential for adenoviral replication under control of a first urothelial cell-specific TRE, and a second adenoviral gene essential for adenoviral replication under control of a second urothelial cell-specific TRE. The first and the second urothelial cell-specific TREs may or may not be substantially identical to one another. By "substantially identical" is meant a requisite degree of sequence identity
10 between the two TREs. The degree of sequence identity between these TREs is at least about 80%, preferably at least about 85%, 90%, 95%, 98%, or 100%. Sequence identity can be determined by a sequence comparison using, i.e., sequence alignment programs that are known in the art, such as those described in *Current Protocols in Molecular Biology* (F.M. Ausubel et al., eds., 1987) Supplement 30, section 7.7.18, Table 7.7.1 A preferred alignment program is ALIGN Plus (Scientific and
15 Educational Software, Pennsylvania), preferably using default parameters. Alternatively, hybridization under stringent conditions can also indicate degree of sequence identity. Adenoviral constructs in which the first and second urothelial cell-specific TREs are substantially identical, particularly if these TREs control
20 transcription of early genes (such as E1A and E1B), may display an instability which may be desirable in certain contexts, such as when an automatic "self-destruction" property can shut down the virus, thereby controlling the degree of propagation.

Stringent conditions for both DNA/DNA and DNA/RNA hybridization are as
25 described by Sambrook et al. *Molecular Cloning, A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989, herein incorporated by reference. For example, see page 7.52 of Sambrook et al. An

example of stringent hybridization conditions is 68°C, 0.2 x SSC other examples are provided herein.

In other embodiments, the adenoviral vector comprises an adenoviral gene essential for adenoviral replication under control of a first urothelial cell-specific TRE, and a transgene under control of a second urothelial cell-specific TRE. The first and the second urothelial cell-specific TREs may or may not be substantially identical to one another.

The size of urothelial cell-specific TREs will be determined in part by the capacity of the adenoviral vector, which in turn depends upon the contemplated form of the vector (see below). Generally a minimal size is preferred, as this provides potential room for insertion of other sequences which may be desirable, such as transgenes (discussed below) or additional regulatory sequences. However, if no additional sequences are contemplated, or if, for example, an adenoviral vector will be maintained and delivered free of any viral packaging constraints, a larger urothelial cell-specific TRE may be used as long as the resultant adenoviral vector is rendered replication-competent.

If no adenovirus sequences have been deleted, an adenoviral vector can be packaged with extra sequences totaling up to about 5% of the genome size, or approximately 1.8 kb. If non-essential sequences are removed from the adenovirus genome, then an additional 4.6 kb of insert can be tolerated (i.e., a total of about 1.8 kb plus 4.6 kb, which is about 6.4 kb). Examples of non-essential adenoviral sequences that can be deleted are E3 and E4 (as long as the E4 ORF6 is maintained). A urothelial cell-specific TRE will comprise a polynucleotide sequence of about 3.5 kb, more preferably smaller fragments which are shown to be functional in controlling transcription in a urothelial cell-specific manner.

In order to minimize non-specific replication, endogenous (i.e., adenovirus) TREs should preferably be removed. This would also provide more room for inserts in an adenoviral vector, which may be of special concern if an adenoviral vector will

be packaged as a virus (see below). Even more importantly, deletion of endogenous TREs would prevent a possibility of a recombination event whereby a urothelial cell-specific TRE is deleted and the endogenous TRE assumes transcriptional control of its respective adenovirus coding sequences (thus allowing non-specific replication).

5 In one embodiment, an adenoviral vector of the invention is constructed such that the endogenous transcription control sequences of an adenoviral gene(s) are deleted and replaced by a urothelial cell-specific TRE. However, endogenous TREs may also be maintained in the adenovirus vector(s), provided that sufficient cell-specific replication preference is preserved. These embodiments can be constructed by
10 providing a urothelial cell-specific TRE in addition to the endogenous TREs, preferably with the urothelial cell-specific TRE intervening between the endogenous TREs and the replication gene coding segment.

Accordingly, in some embodiments, the E1A promoter is inactivated. In other
15 embodiments, E1A enhancer I is inactivated. In some embodiments, the E1A promoter is inactivated and the E1A enhancer I is inactivated. In other embodiments, an internal ribosome entry site (IRES) is inserted upstream of E1B (with the E1B promoter present or not present), and urothelial cell-specific TRE is operably linked to E1A. In still other embodiments, an internal ribosome entry site (IRES) is inserted
20 upstream of E1B, and urothelial cell-specific TRE is operably linked to E1A, which may or may not maintain the E1A promoter and/or enhancer I (i.e., the E1A promoter and/or enhancer I may be, but not necessarily be, deleted). In other embodiments, the 19-kDa region of E1B is deleted. For adenovirus vectors comprising a second gene under control of an IRES, it is preferred that the endogenous promoter of a gene under translational control of an IRES be deleted so that the endogenous promoter
25 does not interfere with transcription of the second gene. It is preferred that the second gene be in frame with the IRES if the IRES contains an initiation codon. If an initiation codon, such as ATG, is present in the IRES, it is preferred that the initiation codon of the second gene is removed and that the IRES and the second gene are in

frame. Alternatively, if the IRES does not contain an initiation codon or if the initiation codon is removed from the IRES, the initiation codon of the second gene is used. Such vectors are described in the Examples. IRES are discussed above and are provided in Table 6 and Table 7.

5 With respect to all of the adenovirus embodiments described herein, requisite urothelial cell-specific replication preference is indicated by conducting assays that compare replication of the adenovirus vector in a cell that allow a urothelial cell-specific TRE to function with replication in a non-urothelial cell. Generally, a replication differential of at least 2-fold is preferred; more preferably, at least 5-fold; 10 more preferably, at least 10-fold; more preferably, at least 50-fold; even more preferably, at least 100-fold; still more preferably, at least 200-fold; still more preferably, at least about 400-fold to about 500-fold; even more preferably, at least 1000-fold. The acceptable differential can be determined empirically (using, for example, Northern assays or other assays known in the art or assays described in the 15 Example section) and will depend upon the anticipated use of the adenoviral vector and/or the desired result.

 Suitable target cells are any cell type that allows a urothelial cell-specific TRE to function. Especially preferred are bladder tumor (carcinoma) cells including, but not limited to, transitional cell carcinoma of the bladder, bladder carcinoma in situ 20 cells, and any metastases of the foregoing. Proteins which are produced by urothelial carcinoma cells but not by normal urothelium include the cytokeratin CK-20. Klein et al. (1998) *Cancer* 82:349-354; GenBank Accession No. X73502; Swiss-Prot Accession No. P35900. Production of bladder cancer cell-specific proteins such as CK-20 can be measured using assays standard in the art, such as RIA, ELISA or 25 Western blots (immunoassays) to determine levels of CK-20 protein production or Northern blots or PCR to determine levels of CK-20 mRNA production. Alternatively, such cells can be identified and/or characterized by their ability to

transcriptionally activate a urothelial cell-specific TRE (i.e., allow a urothelial cell-specific TRE to function).

Any of the various serotypes of adenovirus can be used, such as Ad2, Ad5, Ad12, and Ad40. For purposes of illustration, the serotype Adenovirus 5 (Ad5) is exemplified herein.

The E1A gene is expressed immediately after viral infection (0-2 hours) and before any other viral genes. E1A protein acts as a trans-acting positive-acting transcriptional regulatory factor, and is required for the expression of the other early viral genes E1B, E2, E3, E4, and the promoter-proximal major late genes. Despite the nomenclature, the promoter proximal genes driven by the major late promoter are expressed during early times after Ad5 infection. Flint (1982) *Biochem. Biophys. Acta* 651:175-208; Flint (1986) *Advances Virus Research* 31:169-228; Grand (1987) *Biochem. J.* 241:25-38. In the absence of a functional E1A gene, viral infection does not proceed, because the gene products necessary for viral DNA replication are not produced. Nevins (1989) *Adv. Virus Res.* 31:35-81. The transcription start site of Ad5 E1A is at 498 and the ATG start site of the E1A protein is at nt 560 in the virus genome.

The E1B protein functions in *trans* and is necessary for transport of late mRNA from the nucleus to the cytoplasm. Defects in E1B expression result in poor expression of late viral proteins and an inability to shut off host cell protein synthesis. The promoter of E1B has been implicated as the defining element of difference in the host range of Ad40 and Ad5: clinically Ad40 is an enterovirus, whereas Ad5 causes acute conjunctivitis. Bailey, Mackay et al. (1993) *Virology* 193:631; Bailey et al. (1994) *Virology* 202:695-706. E1B proteins are also necessary to overcome restrictions imposed on viral replication by the host cell cycle and also to reduce the apoptotic effects of E1A. Goodrum et al. (1997) *J. Virology* 71:548-561. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box.

Adenovirus E1B 19-kDa (19K) protein is a potent inhibitor of apoptosis and cooperates with E1A to produce oncogenic transformation of primary cells (Rao, et al., 1992, *Cell Biology*, 89:7742-7746). During productive adenovirus infection, E1A stimulates host cell DNA synthesis, thereby causing cells to aberrantly go through the cell cycle. In response to cell cycle deregulation, the host cell undergoes apoptosis. As a defense mechanism, the E1B 19-kDa protein inhibits this E1A-induced apoptosis and allows assembly of viral progeny to be completed before the cell commits suicide. E1B 19-kDa conducts anti-apoptotic function by multiple mechanisms. E1B 19-kDa inhibits the apoptosis of multiple stimuli, including E1a, p53 and TNF, for example. According to wild-type Ad5, the E1B 19-kDa region is located between nucleotide 1714 and nucleotide 2244. The E1B 19-kDa region has been described in, for example, Rao *et al.*, *Proc. Natl. Acad. Sci. USA*, 89:7742-7746.

The E2 region of adenovirus codes for proteins related to replication of the adenoviral genome, including the 72-kDa DNA-binding protein, the 80-kDa precursor terminal protein and the viral DNA polymerase. The E2 region of Ad5 is transcribed in a rightward orientation from two promoters, termed E2 early and E2 late, mapping at 76.0 and 72.0 map units, respectively. While the E2 late promoter is transiently active during late stages of infection and is independent of the E1A transactivator protein, the E2 early promoter is crucial during the early phases of viral replication.

The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site.

For a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Microbiol. and Immunol.* (1995) 199 part 3:177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation.

However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 kDa protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kDa protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a UP-TRE having SpeI ends into the SpeI site in the 1-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow urothelial cell-restricted expression of E2 transcripts.

The E4 gene produces a number of transcription products. The E4 region codes for two polypeptides which are responsible for stimulating the replication of viral genomic DNA and for stimulating late gene expression. The protein products of open reading frames (ORFs) 3 and 6 can both perform these functions by binding the 55-kDa protein from E1B and heterodimers of E2F-1 and DP-1. The ORF 6 protein requires interaction with the E1B 55-kDa protein for activity while the ORF 3 protein does not. In the absence of functional protein from ORF 3 and ORF 6, plaques are produced with an efficiency less than 10^{-6} that of wild type virus. To further restrict viral replication to cells which permit a urothelial cell-specific TRE to function, E4 ORFs 1-3 can be deleted, making viral DNA replication and late gene synthesis dependent on E4 ORF 6 protein. By combining such a vector with sequences in which the E1B region is regulated by a urothelial cell-specific TRE, a virus can be obtained in which both the E1B function and E4 function are dependent on a urothelial cell-specific TRE driving E1B.

The major late genes relevant to the subject invention are L1, L2, L3, L4 and L5 which encode proteins of the Ad5 virus virion. All of these genes (typically coding for structural proteins) are probably required for adenoviral replication. The late genes are all under the control of the major late promoter (MLP), which is located in Ad5 at about +5986 to about +6048.

Transgenes under transcriptional control of a urothelial cell-specific TRE

Various other replication-competent adenovirus vectors can be made according to the present invention in which, in addition to having a single or multiple adenovirus gene(s) under control of a urothelial cell-specific TRE, a transgene(s) are also under control of a urothelial cell-specific TRE. Transgenes include, but are not limited to, therapeutic transgenes and reporter genes.

Reporter genes

For example, a urothelial cell-specific TRE can be introduced into an adenovirus vector immediately upstream of and operably linked to an early gene such as E1A or E1B, and this construct may also contain at least one other urothelial cell-specific TRE driving expression of a reporter gene. The reporter gene can encode a reporter protein, including, but not limited to, chloramphenicol acetyl transferase (CAT), β -galactosidase (encoded by the *lacZ* gene), luciferase, alkaline phosphatase, a green fluorescent protein, and horse radish peroxidase. For detection of a putative cancer cell(s) in a biological sample, the biological sample may be treated with modified adenoviruses in which a reporter gene (e.g., luciferase) is under control of a urothelial cell-specific TRE. The urothelial cell-specific TRE will be transcriptionally active in cells that allow a urothelial cell-specific TRE to function, and luciferase will be produced. This production will allow detection of urothelial cells, including bladder cancer cells such as transitional cell carcinoma, in, for example, a human host or a biological sample. Alternatively, an adenovirus can be constructed in which a gene encoding a product conditionally required for survival (e.g., an antibiotic resistance marker) is under transcriptional control of a urothelial cell-specific TRE. When this adenovirus is introduced into a biological sample, urothelial cells will become antibiotic resistant. An antibiotic can then be introduced into the medium to kill non-urothelial (e.g., non-cancerous) cells.

Therapeutic transgenes

Transgenes also include genes which may confer a therapeutic effect, such as enhancing cytotoxicity so as to eliminate unwanted target cells. In this way, various genetic capabilities may be introduced into target cells, particularly cancer cells. For example, in certain instances, it may be desirable to enhance the degree and/or rate of cytotoxic activity, due to, for example, the relatively refractory nature or particular aggressiveness of the cancerous target cell. This could be accomplished by coupling the target cell-specific cytotoxic activity with cell-specific expression of, for example, HSV-tk and/or cytosine deaminase (cd), which renders cells capable of metabolizing 5-fluorocytosine (5-FC) to the chemotherapeutic agent 5-fluorouracil (5-FU). Using these types of transgenes may also confer a bystander effect.

Other desirable transgenes that may be introduced via an adenovirus vector(s) include genes encoding cytotoxic proteins, such as the A chains of diphtheria toxin, ricin or abrin (Palmiter et al. (1987) *Cell* 50: 435; Maxwell et al. (1987) *Mol. Cell. Biol.* 7: 1576; Behringer et al. (1988) *Genes Dev.* 2: 453; Messing et al. (1992) *Neuron* 8: 507; Piatak et al. (1988) *J. Biol. Chem.* 263: 4937; Lamb et al. (1985) *Eur. J. Biochem.* 148: 265; Frankel et al. (1989) *Mol. Cell. Biol.* 9: 415), genes encoding a factor capable of initiating apoptosis, sequences encoding antisense transcripts or ribozymes, which among other capabilities may be directed to mRNAs encoding proteins essential for proliferation, such as structural proteins, or transcription factors; viral or other pathogenic proteins, where the pathogen proliferates intracellularly; genes that encode an engineered cytoplasmic variant of a nuclease (e.g. RNase A) or protease (e.g. aprotinin, papain, proteinase K, carboxypeptidase, etc.), or encode the Fas gene, and the like. Other genes of interest include cytokines, antigens, transmembrane proteins, and the like, such as IL-1, -2, -6, -12, GM-CSF, G-CSF, M-CSF, IFN- α , - β , - γ , TNF- α , - β , TGF- α , - β , NGF, and the like. The positive effector genes could be used in an earlier phase, followed by cytotoxic activity due to replication.

E3-containing urothelial cell-specific adenoviral vectors

The invention provides urothelial cell-specific adenovirus vectors comprising an E3 region, or a portion of an E3 region, and an adenoviral gene under transcriptional control of a urothelial cell-specific TRE. Preferably, the vectors are replication-competent. Inclusion of the E3 region of adenovirus can enhance cytotoxicity of the urothelial cell-specific adenoviral vectors of the present invention. Adenoviral vectors containing an E3 region may maintain their high level of specificity and can be (a) significantly more cytotoxic; (b) produce higher virus yield including extracellular virus yield; (c) form larger plaques; (d) produce rapid cell death; and (e) kill tumors more efficiently *in vivo* than vectors lacking the E3 region.

The adenoviral vectors of this invention may contain the E3 region or a portion of the E3 region. It is understood that, as inclusion of E3 confers observable and measurable functionality on the adenoviral vectors, for example, increased replication and production, functionally equivalent (in which functionality is essentially maintained, preserved, or even enhanced or diminished) variants of E3 may be constructed. For example, portions of E3 may be used. As is explained in the definition of "portion" of E3, a portion may be, non-inclusively, either of the following: (a) deletion, preferably at the 3' end; (b) inclusion of one or more various open reading frames of E3. Five proteins which are encoded by the Ad-E3 region have been identified and characterized: (1) a 19-kDa glycoprotein (gp19k) is one of the most abundant adenovirus early proteins, and is known to inhibit transport of the major histocompatibility complex class I molecules to the cell surface, thus impairing both peptide recognition and clearance of Ad-infected cells by cytotoxic T lymphocytes (CTLs); (2) E3 14.7k protein and the E3 10.4k/14.5k complex of proteins inhibit the cytotoxic and inflammatory responses mediated by tumor necrosis factor (TNF); (3) E3 10.4k/14.5k protein complex down regulates the epidermal growth factor receptor, which may inhibit inflammation and activate quiescent

infected cells for efficient virus replication; (4) E3 11.6k protein (adenoviral death protein, ADP) from adenovirus 2 and 5 appears to promote cell death and release of virus from infected cells. The functions of three E3-encoded proteins -- 3.6k, 6.7k and 12.5k -- are unknown. A ninth protein having a molecular weight of 7.5 kDa has been postulated to exist, but has not been detected in cells infected with wild-type adenovirus. Wold et al. (1995) *Curr. Topics Microbiol. Immunol.* 199:237-274. The E3 region is schematically depicted in FIG. 13. These intact, portions, or variants of E3 may be readily constructed using standard knowledge and techniques in the art. Preferably, an intact E3 region is used.

In the adenovirus vectors of the present invention, E3 may or may not be under transcriptional control of native adenoviral transcriptional control element(s). The E3 promoter is located within the coding sequence for virion protein VIII, an essential protein which is highly conserved among adenovirus serotypes. In some embodiments, E3 is under transcriptional control of a heterologous TRE, including, but not limited to, a urothelial cell-specific TRE. Accordingly, in one embodiment, the invention provides an adenoviral vector, preferably replication competent, that comprises E3 region (or a portion of E3) under transcriptional control of a urothelial cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a native adenoviral TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a urothelial cell-specific TRE. In other embodiments, the E3 region is under transcriptional control of a urothelial cell-specific TRE, and the vector further comprises an adenoviral gene essential for replication under transcriptional control of a urothelial cell-specific TRE.

Forms and Administration of Adenoviral Vectors

The adenoviral vectors can be used in a variety of forms, including, but not limited to, naked polynucleotide (usually DNA) constructs. Adenoviral vectors can, alternatively, comprise polynucleotide constructs that are complexed with agents to facilitate entry into cells, such as cationic liposomes or other cationic compounds

such as polylysine; packaged into infectious adenovirus particles (which may render the adenoviral vector(s) more immunogenic); packaged into other particulate viral forms such as HSV or AAV; complexed with agents (such as PEG) to enhance or dampen an immune response; complexed with agents that facilitate *in vivo* transfection, such as DOTMA™, DOTAP™, and polyamines.

If an adenoviral vector comprising an adenovirus polynucleotide is packaged into a whole adenovirus (including the capsid), the adenovirus itself may also be selected to further enhance targeting. For example, adenovirus fibers mediate primary contact with cellular receptor(s) aiding in tropism. See, e.g., Amberg et al. (1997) *Virology* 227:239-244. If a particular subgenus of an adenovirus serotype displayed tropism for a target cell type and/or reduced affinity for non-target cell types, such subgenus(or subgenera) could be used to further increase cell-specificity of cytotoxicity and/or cytolysis.

The adenoviral vectors may be delivered to the target cell in a variety of ways, including, but not limited to, liposomes, general transfection methods that are well known in the art, such as calcium phosphate precipitation, electroporation, direct injection, and intravenous infusion. The means of delivery will depend in large part on the particular adenoviral vector (including its form) as well as the type and location of the target cells (i.e., whether the cells are *in vitro* or *in vivo*).

If used in packaged adenoviruses, adenovirus vectors may be administered in an appropriate physiologically acceptable carrier at a dose of about 10^4 to about 10^{14} . The multiplicity of infection will generally be in the range of about 0.001 to 100. If administered as a polynucleotide construct (i.e., not packaged as a virus) about 0.01 μ g to about 1000 μ g of an adenoviral vector can be administered. The adenoviral vector(s) may be administered one or more times, depending upon the intended use and the immune response potential of the host or may be administered as multiple, simultaneous injections. If an immune response is undesirable, the immune response may be diminished by employing a variety of immunosuppressants, so as to

permit repetitive administration, without a strong immune response. If packaged as another viral form, such as HSV, an amount to be administered is based on standard knowledge about that particular virus (which is readily obtainable from, for example, published literature) and can be determined empirically.

5 ***Host Cells, Compositions and Kits***

The present invention also provides host cells comprising (i.e., transformed with) the adenoviral vectors described herein. Both prokaryotic and eukaryotic host cells can be used as long as sequences requisite for maintenance in that host, such as appropriate replication origin(s), are present. For convenience, selectable markers are also provided. Host systems are known in the art and need not be described in detail herein. Prokaryotic host cells include bacterial cells, for example, *E. coli*, *B. subtilis*, and mycobacteria. Among eukaryotic host cells are yeast, insect, avian, plant, *C. elegans* (or nematode) and mammalian host cells. Examples of fungi (including yeast) host cells are *S. cerevisiae*, *Kluyveromyces lactis* (*K. lactis*), species of *Candida* including *C. albicans* and *C. glabrata*, *Aspergillus nidulans*, *Schizosaccharomyces pombe* (*S. pombe*), *Pichia pastoris*, and *Yarrowia lipolytica*. Examples of mammalian cells are cultured human urothelial cells (HUC), KU-1, MYP3 (a non-tumorigenic rat urothelial cell line), 804G (rat bladder carcinoma cell line), HCV-29, UM-UC-3, SW780, RT4, HL60, KG-1, and KG-1A. COS cells, mouse L cells, LNCaP cells, Chinese hamster ovary (CHO) cells, human embryonic kidney (HEK) cells, and African green monkey cells. *Xenopus laevis* oocytes, or other cells of amphibian origin, may also be used.

The present invention also includes compositions, including pharmaceutical compositions, containing the adenoviral vectors described herein. Such compositions are useful for administration *in vivo*, for example, when measuring the degree of transduction and/or effectiveness of cell killing in an individual. Compositions can comprise an adenoviral vector(s) of the invention and a suitable solvent, such as a physiologically acceptable buffer. These are well known in the art. In other

embodiments, these compositions further comprise a pharmaceutically acceptable excipient. These compositions, which can comprise an effective amount of an adenoviral vector of this invention in a pharmaceutically acceptable excipient, are suitable for systemic or local administration to individuals in unit dosage forms, sterile parenteral solutions or suspensions, sterile non-parenteral solutions or oral solutions or suspensions, oil in water or water in oil emulsions and the like. Formulations for parenteral and nonparenteral drug delivery are known in the art and are set forth in *Remington's Pharmaceutical Sciences*, 19th Edition, Mack Publishing (1995). Compositions also include lyophilized and/or reconstituted forms of the adenoviral vectors (including those packaged as a virus, such as adenovirus) of the invention.

The present invention also encompasses kits containing an adenoviral vector(s) of this invention. These kits can be used for diagnostic and/or monitoring purposes, preferably monitoring. Procedures using these kits can be performed by clinical laboratories, experimental laboratories, medical practitioners, or private individuals. Kits embodied by this invention allow someone to detect the presence of bladder cancer cells in a suitable biological sample, such as biopsy specimens.

The kits of the invention comprise an adenoviral vector described herein in suitable packaging. The kit may optionally provide additional components that are useful in the procedure, including, but not limited to, buffers, developing reagents, labels, reacting surfaces, means for detection, control samples, instructions, and interpretive information.

Preparation of the adenovirus vectors of the invention

The adenovirus vectors of this invention can be prepared using recombinant techniques that are standard in the art. Generally, a urothelial cell-specific TRE is inserted 5' to the adenoviral gene of interest, preferably an adenoviral replication gene, more preferably one or more early replication genes (although late gene(s) can be used). A urothelial cell-specific TRE can be prepared using oligonucleotide

synthesis (if the sequence is known) or recombinant methods (such as PCR and/or restriction enzymes). Convenient restriction sites, either in the natural adeno-DNA sequence or introduced by methods such as PCR or site-directed mutagenesis, provide an insertion site for a urothelial cell-specific TRE. Accordingly, convenient restriction sites for annealing (i.e., inserting) a urothelial cell-specific TRE can be engineered onto the 5' and 3' ends of a UP-TRE using standard recombinant methods, such as PCR.

Polynucleotides used for making adenoviral vectors of this invention may be obtained using standard methods in the art, such as chemical synthesis, recombinant methods and/or obtained from biological sources.

Adenoviral vectors containing all replication-essential elements, with the desired elements (e.g., E1A) under control of a urothelial cell-specific TRE, are conveniently prepared by homologous recombination or *in vitro* ligation of two plasmids, one providing the left-hand portion of adenovirus and the other plasmid providing the right-hand region, one or more of which contains at least one adenovirus gene under control of a urothelial cell-specific TRE. If homologous recombination is used, the two plasmids should share at least about 500 bp of sequence overlap. Each plasmid, as desired, may be independently manipulated, followed by cotransfection in a competent host, providing complementing genes as appropriate, or the appropriate transcription factors for initiation of transcription from a urothelial cell-specific TRE for propagation of the adenovirus. Plasmids are generally introduced into a suitable host cell such as 293 cells using appropriate means of transduction, such as cationic liposomes. Alternatively, *in vitro* ligation of the right and left-hand portions of the adenovirus genome can also be used to construct recombinant adenovirus derivative containing all the replication-essential portions of adenovirus genome. Berkner et al. (1983) *Nucleic Acid Research* 11: 6003-6020; Bridge et al. (1989) *J. Virol.* 63: 631-638.

For convenience, plasmids are available that provide the necessary portions of adenovirus. Plasmid pXC.1 (McKinnon (1982) *Gene* 19:33-42) contains the wild-type left-hand end of Ad5. pBHG10 (Bett et al. (1994); Microbix Biosystems Inc., Toronto) provides the right-hand end of Ad5, with a deletion in E3. The deletion in E3 provides room in the virus to insert a 3 kb urothelial cell-specific TRE without deleting the endogenous enhancer/promoter. The gene for E3 is located on the opposite strand from E4 (r-strand). pBHG11 provides an even larger E3 deletion (an additional 0.3 kb is deleted). Bett et al. (1994). Alternatively, the use of pBHGE3 (Microbix Biosystems, Inc.) provides the right hand end of Ad5, with a full-length of E3.

For manipulation of the early genes, the transcription start site of Ad5 E1A is at 498 and the ATG start site of the E1A coding segment is at 560 in the virus genome. This region can be used for insertion of a urothelial cell-specific TRE. A restriction site may be introduced by employing polymerase chain reaction (PCR), where the primer that is employed may be limited to the Ad5 genome, or may involve a portion of the plasmid carrying the Ad5 genomic DNA. For example, where pBR322 is used, the primers may use the EcoRI site in the pBR322 backbone and the XbaI site at nt 1339 of Ad5. By carrying out the PCR in two steps, where overlapping primers at the center of the region introduce a nucleotide sequence change resulting in a unique restriction site, one can provide for insertion of urothelial cell-specific TRE at that site. Example 3 provides a more detailed description of an adenoviral vector in which E1A is under urothelial cell-specific TRE control.

A similar strategy may also be used for insertion of a urothelial cell-specific TRE element to regulate E1B. The E1B promoter of Ad5 consists of a single high-affinity recognition site for Spl and a TATA box. This region extends from Ad5 nt 1636 to 1701. By insertion of a urothelial cell-specific TRE in this region, one can provide for cell-specific transcription of the E1B gene. By employing the left-hand region modified with the cell-specific response element regulating E1A, as the

template for introducing a urothelial cell-specific TRE to regulate E1B, the resulting adenovirus vector will be dependent upon the cell-specific transcription factors for expression of both E1A and E1B. In some embodiments, the E1B 19-kDa region is deleted. For a deletion of the genomic region encoding the E1B 19-kDa product in an adenovirus construct, nucleotides encoding the 19-kDa region are deleted. In Ad5, a deletion of the 261 base pairs between nucleotide 1713 and nucleotide 1974 results in a deletion of the genomic region encoding the E1B 19-kDa product. Examples 1, 3, and 5 provide a more detailed description of how such constructs can be prepared.

Similarly, a urothelial cell-specific TRE can be inserted upstream of the E2 gene to make its expression cell-specific. The E2 early promoter, mapping in Ad5 from 27050-27150, consists of a major and a minor transcription initiation site, the latter accounting for about 5% of the E2 transcripts, two non-canonical TATA boxes, two E2F transcription factor binding sites and an ATF transcription factor binding site (for a detailed review of the E2 promoter architecture see Swaminathan et al., *Curr. Topics in Micro. and Immunol.* (1995) 199(part 3):177-194.

The E2 late promoter overlaps with the coding sequences of a gene encoded by the counterstrand and is therefore not amenable for genetic manipulation. However, the E2 early promoter overlaps only for a few base pairs with sequences coding for a 33 kD protein on the counterstrand. Notably, the SpeI restriction site (Ad5 position 27082) is part of the stop codon for the above mentioned 33 kD protein and conveniently separates the major E2 early transcription initiation site and TATA-binding protein site from the upstream transcription factor binding sites E2F and ATF. Therefore, insertion of a urothelial cell-specific TRE having SpeI ends into the SpeI site in the 1-strand would disrupt the endogenous E2 early promoter of Ad5 and should allow urothelial cell-restricted expression of E2 transcripts.

For E4, one must use the right hand portion of the adenovirus genome. The E4 transcription start site is predominantly at about nt 35605, the TATA box at about nt 35631 and the first AUG/CUG of ORF1 is at about nt 35532. Virtanen et al. (1984)

5 *J. Virol.* 51: 822-831. Using any of the above strategies for the other genes, a UP-TRE may be introduced upstream from the transcription start site. For the construction of full-length adenovirus with a urothelial cell-specific TRE inserted in the E4 region, the co-transfection and homologous recombination are performed in W162 cells (Weinberg et al. (1983) *Proc. Natl. Acad. Sci.* 80:5383-5386) which provide E4 proteins *in trans* to complement defects in synthesis of these proteins.

10 Adenoviral constructs containing an E3 region can be generated as described in Example 3, wherein homologous recombination between an E3-containing adenoviral plasmid, for example, BHGE3 (Microbix Biosystems Inc., Toronto) and a non-E3-containing adenoviral plasmid, is carried out.

15 Alternatively, an adenoviral vector comprising an E3 region can be introduced into cells, for example 293 cells, along with an adenoviral construct or an adenoviral plasmid construct, where they can undergo homologous recombination to yield adenovirus containing an E3 region. In this case, the E3-containing adenoviral vector and the adenoviral construct or plasmid construct contain complementary regions of adenovirus, for example, one contains the left-hand and the other contains the right-hand region, with sufficient sequence overlap as to allow homologous recombination.

20 Alternatively, an E3-containing adenoviral vector of the invention can be constructed using other conventional methods including standard recombinant methods (e.g., using restriction nucleases and/or PCR), chemical synthesis, or a combination of any of these. Further, deletions of portions of the E3 region can be created using standard techniques of molecular biology.

Methods of packaging adenovirus polynucleotides into adenovirus particles are known in the art and are described in the Examples.

Methods using the adenovirus vectors of the invention

The subject vectors can be used for a wide variety of purposes, which will vary with the desired or intended result. Accordingly, the present invention includes methods using the adenoviral vectors described above.

5 In one embodiment, methods are provided for conferring selective cytotoxicity in cells that allow a urothelial cell-specific TRE to function, preferably urothelial cells, comprising contacting such cells with an adenovirus vector described herein. Cytotoxicity can be measured using standard assays in the art, such as dye exclusion, ³H-thymidine incorporation, and/or lysis.

10 In another embodiment, methods are provided for propagating an adenovirus specific for cells which allow a urothelial cell-specific TRE to function, preferably urothelial cells, preferably bladder cancer cells. These methods entail combining an adenovirus vector with the cells, whereby said adenovirus is propagated.

15 Another embodiment provides methods for killing cells that allow a urothelial cell-specific TRE to function in a mixture of cells, comprising combining the mixture of cells with an adenovirus vector of the present invention. The mixture of cells is generally a mixture of normal cells and cancerous cells that allow a urothelial cell-specific TRE to function, and can be an *in vivo* mixture or *in vitro* mixture.

20 The invention also includes methods for detecting cells which allow a urothelial cell-specific TRE to function, such as bladder cancer cells, in a biological sample. These methods are particularly useful for monitoring the clinical and/or physiological condition of an individual (i.e., mammal), whether in an experimental or clinical setting. In one method, cells of a biological sample are contacted with an adenovirus vector, and replication of the adenoviral vector is detected. Alternatively,
25 the sample can be contacted with an adenovirus in which a reporter gene is under control of a urothelial cell-specific TRE. When such an adenovirus is introduced into a biological sample, expression of the reporter gene indicates the presence of cells that allow a urothelial cell-specific TRE to function. Alternatively, an adenovirus can be constructed in which a gene conditionally required for cell survival is placed under

control of a urothelial cell-specific TRE. This gene may encode, for example, antibiotic resistance. Later the biological sample is treated with an antibiotic. The presence of surviving cells expressing antibiotic resistance indicates the presence of cells capable of urothelial cell-specific TRE function. A suitable biological sample is one in which cells that allow a urothelial cell-specific TRE to function, such as bladder cancer cells, may be or are suspected to be present. Generally, in mammals, a suitable clinical sample is one in which cancerous cells that allow a urothelial cell-specific TRE to function, such as bladder carcinoma cells, are suspected to be present. Such cells can be obtained, for example, by needle biopsy or other surgical procedure. Cells to be contacted may be treated to promote assay conditions, such as selective enrichment, and/or solubilization. In these methods, cells that allow a urothelial cell-specific TRE to function can be detected using *in vitro* assays that detect adenoviral proliferation, which are standard in the art. Examples of such standard assays include, but are not limited to, burst assays (which measure virus yield) and plaque assays (which measure infectious particles per cell). Propagation can also be detected by measuring specific adenoviral DNA replication, which are also standard assays.

The invention also provides methods of modifying the genotype of a target cell, comprising contacting the target cell with an adenovirus vector described herein, wherein the adenoviral vector enters the cell.

The invention further provides methods of suppressing tumor cell growth, preferably a tumor cell that allows a urothelial cell-specific TRE to function, comprising contacting a tumor cell with an adenoviral vector of the invention such that the adenoviral vector enters the tumor cell and exhibits selective cytotoxicity for the tumor cell. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The invention also provides methods of lowering the levels of a tumor cell marker in an individual, comprising administering to the individual an adenoviral vector of the present invention, wherein the adenoviral vector is selectively cytotoxic toward cells that allow a urothelial cell-specific TRE to function. Tumor cell markers include, but are not limited to, CK-20. Methods of measuring the levels of a tumor cell marker are known to those of ordinary skill in the art and include, but are not limited to, immunological assays, such as enzyme-linked immunosorbent assay (ELISA), using antibodies specific for the tumor cell marker. In general, a biological sample is obtained from the individual to be tested, and a suitable assay, such as an ELISA, is performed on the biological sample. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The invention also provides methods of treatment, in which an effective amount of an adenoviral vector(s) described herein is administered to an individual. Treatment using an adenoviral vector(s) is indicated in individuals with bladder cancer as described above. Also indicated are individuals who are considered to be at risk for developing bladder cancer (including single cells), such as those who have had disease which has been resected and those who have had a family history of bladder cancer. Determination of suitability of administering adenoviral vector(s) of the invention will depend, inter alia, on assessable clinical parameters such as serological indications and histological examination of tissue biopsies. Generally, a pharmaceutical composition comprising an adenoviral vector(s) in a pharmaceutically acceptable excipient is administered. Pharmaceutical compositions are described above. For these methods, the adenoviral vector may or may not be used in conjunction with other treatment modalities for tumor suppression, such as chemotherapeutic agents (such as those listed below), radiation and/or antibodies.

The amount of adenoviral vector(s) to be administered will depend on several factors, such as route of administration, the condition of the individual, the degree of aggressiveness of the disease, the particular urothelial cell-specific TRE employed, and the particular vector construct (i.e., which adenovirus gene(s) is under urothelial cell-specific TRE control), as well as whether the adenoviral vector is used in conjunction with other treatment modalities.

If administered as a packaged adenovirus, from about 10^4 to about 10^{14} , preferably from about 10^4 to about 10^{12} , more preferably from about 10^4 to about 10^{10} . If administered as a polynucleotide construct (i.e., not packaged as a virus), about 0.01 μ g to about 100 μ g can be administered, preferably 0.1 μ g to about 500 μ g, more preferably about 0.5 μ g to about 200 μ g. More than one adenoviral vector can be administered, either simultaneously or sequentially. Administrations are typically given periodically, while monitoring any response. Administration can be given, for example, intratumorally, intravenously or intraperitoneally.

The adenoviral vectors of the invention can be used alone or in conjunction with other active agents, such as chemotherapeutics, that promote the desired objective. Examples of chemotherapeutics which are suitable for suppressing bladder tumor growth are BGC (bacillus Calmett-Guerin); mitomycin-C; cisplatin; thiotepa; doxorubicin; methotrexate; paclitaxel (TAXOLTM); ifosfamide; gallium nitrate; gemcitabine; carboplatin; cyclophosphamid; vinblastine; vincristin; fluorouracil; etoposide; bleomycin. Examples of combination therapies include (CISCA (cyclophosphamide, doxorubicin, and cisplatin); CMV (cisplatin, methotrexate, vinblastine); MVMJ (methotrexate, vinblastine, mitoxantrone, carboplatin); CAP (cyclophosphamide, doxorubicin, cisplatin); MVAC (methotrexate, vinblastine, doxorubicin, cisplatin). Radiation may also be combined with chemotherapeutic agent(s), for example, radiation with cisplatin. Administration of the chemotherapeutic agents is generally intravesical (directly into the bladder) or intravenous.

The following examples are provided to illustrate but not limit the invention.

EXAMPLES

5 **Example 1: Mouse and human uroplakin-derived urothelial cell-specific TRE constructs**

A 3.6 kb portion of 5'-flanking DNA a mouse UPII was amplified from mouse genomic DNA using PCR with primers 66.119.1 and 66.119.2.

10 66.119.1 (5'-ACCGGTCTCGAGGATCTCGGCCCTCTTTC-3', SEQ ID NO: _____)

66.119.2 (5'-ACCGGTACTGCGCTGGGACTGGATCC-3', SEQ ID NO: _____)

15 The amplified fragment was purified, then "TA" cloned by ligation into pGEM-T (Promega) to created plasmid CN568. The entire insert was amplified from CN568 with primers 100.24.1

(5'-AAGCTTACCGGTACTGCGCTGGGACTGGATCCTG-3', SEQ ID NO: _____) and 100.27.1

20 (5'-ACCATGGACCGGTCTCGAGGATCTCGGCCCTCTTTC-3', SEQ ID NO: _____), purified, and ligated into pGEM-T to create plasmid CP616. CP616 was digested with HindIII and SpeI, blunted and ligated into pGL3-Basic (Promega) which had been digested with HindIII and MluI and blunted, creating plasmid CP620. CP620 contains the 3.6 kb mUPII 5'-flanking DNA (nucleotides -3531 to +60) in operable linkage with the *luc+* gene.

25 Plasmids CP619 and CP618 were created with a similar strategy. A 1.0 kb fragment (-965 to +1) of the 5' flanking DNA from the mUPII gene with primers 100.24.1 and 100.24.3

(5'-ACCATGGACCGGTACGTACCCAATCTGTTGTCCCAG-3', SEQ ID NO: _____) and a 600 bp fragment (-587 to +1) of the 5'-flanking DNA from the mUPII gene was

amplified with 100.24.1 and 100.24.2 (5'-
ACCATGGACCGGTCAGCTAGCCTTGCTGGACTGGAC-3', SEQ ID NO:____).
Each fragment was purified then TA cloned into pGEM-T, creating CP615 and
CP614, respectively. The 1.0 and 0.6 kb fragments were excised from CP615 and
CP614 by digestion with SpeI, purified, blunted and digested with HindIII, then
ligated into pGL3-Basic (Promega) which had been digested with MluI, blunted, and
digested with HindIII, creating plasmids CP619 and CP618, respectively.

5' flanking DNA from human UPII was isolated from human genomic DNA
using a Human GenomeWalker kit from Clontech (Palo Alto, CA) according to the
manufacturer's instructions. Briefly, a first PCR reaction was performed using the
AP1 primer supplied in the kit in combination with a hUPII-specific 3' primer,
100.84.1, which is complementary to positions +24 to +47 of the hUPII gene (5'-
AAGAATCAGGATCAAGGGCAAGTC-3', SEQ ID NO:____). The product of
the first PCR reaction was then amplified a second time using a nested set of primers
consisting of AP2 (supplied in the kit) and 100.84.2, which is complementary to
positions +3 to -22 of the hUPII gene (5'-AATGCTGGGCTGGGAGGTGGAATAG-
3', SEQ ID NO:____). Five major amplification products from the second PCR
reaction were TA cloned into pGEM-T. One clone, #7, were identified as containing
a 2.2 kb segment of DNA from the 5'-flanking region of hUPII. The 2.2 kb segment
was subcloned by amplification using primers 100.113.1
(5'-AGGGGTACCCACTATAGGGCACGCGTGGT-3', SEQ ID NO:____) and
100.113.2 (5'-ACCCAAGCTTGGGATGCTGGGCTGGGAGGTGG-3', SEQ ID
NO:____), purification, and TA cloning into pGEM-T, creating CP655. The insert was
then excised by digestion with HindIII and SpeI, purified and blunted. The 2.2 (-
2225 to +1) kb fragment from CP655 was cloned into pGL3-Basic which had been
digested with SacII, blunted, and digested with KpnI, creating CP657. A second
clone (#16) contained a 1.0 kb fragment of 5'-flanking DNA. This fragment was
subcloned by amplification with primers 100.113.1 and 100.113.2, purification, and

TA cloning into pGEM-T to generate CP654. The 1.0 kb insert (-965 to +1) was excised from CP654 with KpnI (blunt) then HindIII, and cloned into pGL3-Basic which had been digested with SacII, blunted, and digested with HindIII to create CP656.

5 Additional, smaller fragments (0.6 kb and 0.2 kb) of the 5'-flanking region from hUPII were amplified from using 100.126.3

(5'-ACGAGGGGTACCCACCGGTACCGCATGTGCTCCCTGGCC-3',

SEQ ID NO: __) plus 100.126.1

(5'-AGACCCAAGCTTGGGACCGGTATGCTGGGCTGGGAGGTGG-3',

10 SEQ ID NO: __) and 100.126.2

(5'-ACGAGGGGTACCCACCGGTCCCCCTCCTGGCCTGAGG-3',

SEQ ID NO: __) plus 100.126.1, respectively, purified, and TA cloned into pGEM-T, creating CP658 and CP659, respectively. CP658 and CP659 were each digested with

KpnI and HindIII to excise the 0.6 (-592 to +1) and 0.2 (-211 to +1) kb hUPII 5'-

15 flanking fragments, which were each purified and cloned into pGL3-Basic which had also been digested with KpnI and HindIII, creating CP662 and CP663, respectively.

Two segments of 5'-flanking sequence from human UP1a were cloned by amplifying human genomic DNA with primers 100.82.1

(5'-AGGGGTACCCCGGCCGGTCACACAGCAGGAGAGACAC-3',

20 SEQ ID NO: __) plus 100.82.2

(5'-ACCCAAGCTTGGGCGGCCGCATCCTGGGACACATGAGCAGG-3',

SEQ ID NO: __) and 100.82.2 plus 100.83.1

(5'-AGGGGTACCCCGGCCGCAACCCTGCCTTCGAGGTTC-3', SEQ ID NO: __),

and TA cloning the amplification products into pGEM-T, creating CP646 (1.0 kb

25 fragment) and CP647 (2.0 kb fragment). CP646 and CP647 were each digested with KpnI and HindIII to excise the inserts, which were each purified and cloned into pGL3-Basic which had been KpnI/HindIII digested, creating CP648 and CP649,

respectively. The characteristics of the various plasmids are summarized in Table 1 (Fig. 3).

TABLE 1

Name	Backbone	Insert Size	Species	Gene	Comments
CP655	pGEM-T	2.2 kb	human	UPII	GenomeWalker product
CN568	pGEM-T	3.6 kb	mouse	UPII	
CP614	pGEM-T	0.6 kb	mouse	UPII	Plus restriction sites
CP615	pGEM-T	1.0 kb	mouse	UPII	Plus restriction sites
CP616	pGEM-T	3.6 kb	mouse	UPII	Plus restriction sites
CP618	pGL3-Basic	0.6 kb	mouse	UPII	Plus restriction sites, linked to <i>luc+</i>
CP619	pGL3-Basic	1.0 kb	mouse	UPII	Plus restriction sites, linked to <i>luc+</i>
CP620	pGL3-Basic	3.6 kb	mouse	UPII	Plus restriction sites, linked to <i>luc+</i>
CP646	pGEM-T	1.0 kb	human	UP1a	Plus restriction sites
CP647	pGEM-T	2.0 kb	human	UP1a	Plus restriction sites
CP648	pGL3-Basic	2.0 kb	human	UP1a	Plus restriction sites, linked to <i>luc+</i>
CP649	pGL3-Basic	1.0 kb	human	UP1a	Plus restriction sites, linked to <i>luc+</i>
CP654	pGEM-T	1.0 kb	human	UPII	Plus restriction sites
CP655	pGEM-T	2.2 kb	human	UPII	Plus restriction sites
CP656	pGL3-Basic	1.0 kb	human	UPII	Plus restriction sites, linked to <i>luc+</i>
CP657	pGL3-Basic	2.2 kb	human	UPII	Plus restriction sites, linked to <i>luc+</i>
CP658	pGEM-T	0.6 kb	human	UPII	Plus restriction sites
CP659	pGEM-T	0.2 kb	human	UPII	Plus restriction sites
CP662	pGL3-Basic	0.2 kb	human	UPII	Plus restriction sites, linked to <i>luc+</i>
CP663	pGL3-Basic	0.6 kb	human	UPII	Plus restriction sites, linked to <i>luc+</i>
CP1066	pGL3-Basic	1.9 kb	mouse	UPII	Plus restriction sites, linked to <i>luc+</i>

Example 2: Urothelial cell-specific TRE reporter assays

5 Uroplakin 5'-flanking DNA was tested for the ability to drive urothelial cell-specific expression in a luciferase-based reporter assay. Luciferase expression was assayed in a variety of different cell lines. The cell lines and their sources are listed in Table 2.

TABLE 2

Name	Source
HBL-100	Breast epithelium
HepG2	Hepatocellular carcinoma
KB	Oral epidermoid carcinoma
LoVo	Colon carcinoma
MCF-7	Breast adenocarcinoma
OVCAR	Ovarian adenocarcinoma
PA-1	Ovary teratocarcinoma
RT-4	Transitional bladder cell papilloma
SW1463	Rectal adenocarcinoma
SW780	Bladder cell carcinoma
UM-UC-3	Bladder cell carcinoma
Hep 3B	Hepatoma

10 The cells were plated at 5×10^5 cells per 60 mm dish in complete RPMI 1640 medium and co-transfected with the various reporter constructs and pCMV- β gal (a plasmid carrying the β -galactosidase gene under the control of the CMV promoter) using a cationic lipid reagent (lipofectin). After a four hour incubation with the

plasmid/lipofectin complexes, the medium was removed by aspiration and replaced with fresh RPMI 1640. The cells were incubated for a further forty eight hours at 37° C, then harvested by aspiration of the medium and lysis in 500 µl of lysate buffer (Analytical Luminescence Laboratories). A 50 µl aliquot was assayed for luciferase activity in a microtiter plate-format luminometer (Dynatech Laboratories, Model ML3000). Luciferase activities were normalized to β-gal activity, which was measured using a kit from Tropix (GALACTO-LIGHT™). The results are shown in FIGS. 5-6.

Constructs CP648 and CP649 showed no preferential expression in SW780 cells. However, CP618, CP620, CP662, CP663, CP656 and CP657 showed significant preferential expression in SW780 cells, indicating the presence of at least one urothelial cell-specific TRE in each of these constructs. CP618 and CP620 showed preferential expression in SW780 cells, while CP619 showed little expression in these cells.

Analysis of the data suggests that a minimal urothelial cell-specific promoter is contained within 600 bp of the transcriptional start site of the mUPII gene, but that the hUPII gene urothelial cell-specific promoter extends somewhat further upstream. The data also suggest a negative regulatory element is located between -600 and -1000 bp. The presence of the silencer in the mUPII 5'-flanking DNA and the high expression of CP620 further suggest the presence of a urothelial cell-specific enhancer located more than 1.0 kb from the transcriptional start site.

Example 3: Adenovirus vectors with urothelial cell-specific TREs

A number of plasmid constructs were generated as intermediates for adenovirus type 5 (Ad 5) vector construct. The plasmid constructs were based on plasmid CP321 (Yu et al., 1999, *Cancer Res.* 59:4200-4203), which contains a prostate-specific enhancer inserted at a PinAI site upstream of the E1A gene and at a EagI site upstream of the E1B gene. Constructs were created by inserting various

UPII-derived 5'-flanking DNA sequences into the PinAI and EagI sites and removing the prostate-specific enhancer. Characteristics of the plasmids, all of which lacked the E1A promoter and which contained the E1A enhancer, are summarized in Table 3.

TABLE 3

Name	E1A TRE	E1B TRE
CP656	1.0 kb hUPII	E1B endogenous promoter
CP657	2.4 kb hUPII	E1B endogenous promoter
CP569	3.6 mUPII	E1B endogenous promoter
CP622	0.6 kb mUPII	E1B endogenous promoter
CP623	1.0 kb mUPII	E1B endogenous promoter
CP662	0.2 kb hUPII	E1B endogenous promoter
CP663	0.6 kb hUPII	E1B endogenous promoter
CP664	0.6 kb hUPII	E1B endogenous promoter
CP665	1.0 kb hUPII	E1B endogenous promoter
CP666	0.6 kb mUPII	0.6 kb mUPII
CP667	0.6 kb mUPII	1.0 kb hUPII
CP668	1.0 kb mUPII	0.6 kb mUPII
CP669	1.0 kb mUPII	1.0 kb hUPII
CP670	0.6 kb hUPII	0.6 kb mUPII
CP671	0.6 kb hUPII	1.0 kb mUPII
CP672	1.0 kb hUPII	0.6 kb mUPII
CP673	1.0 kb hUPII	1.0 kb mUPII
CP1086	1.9 kb mUPII	Replaced by IRES
CP1087	1.0 kb hUPII	Replaced by IRES
CP1088	2.2 kb hUPII	Replaced by IRES
CP1089	1.0 kb mUPII	1.0 kb hUPII

Infectious recombinant adenoviral vectors was produced by co-transfecting 293 cells with the UPII 5'-flanking DNA/E1 constructs and an Ad 5 backbone vector (pBHG10 or pBHGE3, Microbix, Inc.) as described in Yu et al. (*id.*). The characteristics of the viral vectors, all of which lack the E1A promoter and retain the E1A enhancer are summarized in Table 4.

TABLE 4

Name	Vector	Ad 5 Vector	E1A TRE	E1B TRE	E3
CV808	CP569	pBHG10	3.6 kb mUPII	E1B endogenous promoter	deleted
CV818	CP622	pBHG10	0.6 kb mUPII	E1B endogenous promoter	deleted
CV819	CP622	pBHGE3	0.6 kb mUPII	E1B endogenous promoter	intact
CV820	CP623	pBHG10	1.0 kb mUPII	E1B endogenous promoter	deleted
CV821	CP623	pBHGE3	1.0 kb mUPII	E1B endogenous promoter	intact
CV822	CP664	pBHG10	0.6 kb hUPII	E1B endogenous promoter	deleted
CV823	CP664	pBHGE3	0.6 kb mUPII	E1B endogenous promoter	intact
CV824	CP665	pBHG10	1.0 kb hUPII	E1B endogenous promoter	deleted
CV825	CP665	pBHGE3	1.0 kb hUPII	E1B endogenous promoter	intact
CV826	CP667	pBHG10	0.6 kb mUPII	1.0 kb hUPII	deleted
CV827	CP667	pBHGE3	0.6 kb mUPII	1.0 kb hUPII	intact
CV828	CP669	pBHG10	1.0 kb mUPII	1.0 kb hUPII	deleted
CV829	CP669	pBHGE3	1.0 kb hUPII	1.0 kb mUPII	intact
CV830	CP672	pBHG10	1.0 kb hUPII	0.6 kb mUPII	deleted
CV831	CP672	pBHGE3	1.0 kb hUPII	0.6 kb mUPII	intact
CV832	CP673	pBHG10	1.0 kb hUPII	1.0 kb mUPII	deleted
CV833	CP673	pBHGE3	1.0 kb hUPII	1.0 kb mUPII	intact

Replication specificity as indicated by virus yield assays. CV808 and CN702

(an Ad 5 variant containing a wild type E1 region and an E3 deletion identical to the E3 deletion in CV808) were tested against a panel of cell lines for viral replication (indicative of lethality) and specificity. Cell lines 293 (the producer line), SW780, UM-UC-3, HBL-100, PA-1, LoVo and Hep3B were plated at 0.5×10^6 per well in 6 well tissue culture plates, incubated for 24 hours at 37° C, then infected with CV808

or CN702 at a multiplicity of infection (MOI) of 2 plaque forming units per cell (PFU/cell) for 4 hours at 37° C. At the end of the infection period, the medium was replaced and the cells were incubated at 37° C for a further 72 hours before harvesting for a viral yield assay as described in Yu et al. (1999) *Cancer Res.* 59:1498-1504. Another experiment compared CV802 and CV829. The results are shown in FIGS 8 and 9.

CV808 and CN702 replicate equally well in bladder transitional cell carcinoma cells (SW780 and UM-UC-3), but burst size for CV808, as compared to CN702, was substantially reduced (*i.e.*, 100 to 1000 fold) in non-bladder cell lines. The data show that CV808 preferentially replicates in bladder cells (*i.e.*, is significantly attenuated in non-bladder tumor cells). The burst size was significantly reduced by more than 100 to 1000- fold in non-bladder carcinoma cells including LoVo, Hep3B, HBL-100 and PA-1. Further, CV829 preferentially replicates in urothelial cell lines SW780, UM-UC3, and RT-4, compared to non-urothelial cell lines G361, MKNI, and PA-1.

Preferential replication of CV829 in SW780 cells versus smooth muscle cells.

A virus yield experiment was performed as described above to compare replication of CV829 in SW780 (urothelial cells) versus bladder smooth muscle cells (Clonetics Corp.; BioWhittaker, Inc.). The results are shown in FIG. 10. CV829 (containing a mUPII TRE driving transcription of E1A and a hUPII TRE driving transcription of E1B) showed marked reduction of replication in primary smooth muscle cells as compared to SW780 cells. Further, this result was confirmed in a CPE (cytotoxicity assay), which showed that CV829 lysed bladder smooth muscle cells significantly less than degree of lysis of SW780.

This is a significant result which indicates that adenovirus containing uroplakin TREs would confine replication to the target cells of interest in the bladder, namely urothelial cells, while displaying very low levels of replication in adjacent smooth muscle cells.

Example 4: Uroplakin adenoviral constructs containing an IRES

A number of E3-containing viral constructs were prepared which contained uroplakin II sequences (mouse and/or human) as well as an EMCV internal ribosome entry site (IRES). The viral constructs are summarized in Table 5. All of these vectors lacked an E1A promoter and retained the E1A enhancer.

The 519 base pair EMCV IRES segment was PCR amplified from Novagen's pCITE vector by primers A/B:

primer A: 5'-GACGTCGACTAATTCCGGTTATTTTCCA

primer B 5'-GACGTCGACATCGTGTTTTTCAAAGGAA (*GTCGAC* is a *S*all site).

The EMCV IRES element was ligated to PCR blunt vector (Invitrogen pCR® blunt vector).

CP1066

The 1.9kb-(-1885 to +1) fragment of mouse UPII from CP620 was digested with *Afl*III (blunted) and *Hind*III and inserted into pGL3-Basic from CP620 which had been digested with *Xho*I (blunted) and *Hind*III to generate CP1066.

CP1086

The 1.9kb mouse UPII insert was digested with *Pin*AI and ligated with CP269 (CMV driving E1A and IRES driving E1B with the deletions of E1A/E1B endogenous promoter) which was similarly cut by *Pin*AI.

CP1087

The 1kb (-1128 to +1) human UPII was digested with PinAI from CP665 and inserted into CP629 which had been cut by PinAI and purified (to elute CMV).

5 ***CP1088***

The 2.2kb (-2225 to +1) human UPII was amplified from CP657 with primer 127.2.1 (5'-AGGACCGGTCACTATAGGGCACGCGTGGT-3') PLUS 127.2.2 (5'-AGGACCGGTGGGATGCTGGGCTGGGAGGTGG-3') and digested with PinAI and ligated with CP629 cut with PinAI.

10 CP627 is an Ad5 plasmid with an internal ribosome entry site (IRES) from encephelomyocarditis virus (EMCV) at the junction of E1A and E1B. First, CP306 (Yu et al., 1999) was amplified with primer pairs 96.74.3/96.74.6 and 96.74.4/96.74.5.

15 The two PCR products were mixed and amplified with primer pairs 96.74.3 and 96.74.5. The resultant PCR product contains a 100bp deletion in E1A-E1B intergenic region and a new SalI site at the junction. EMCV IRES fragment was amplified from pCITE-3a(+) (Novagen) using primers 96.74.1 and 96.74.2. The SalI fragment containing IRES was placed into SalI site to generate CP627 with the bicistronic E1A-IRES-E1B cassette. CP629 is a plasmid with CMV promoter
20 amplified from pCMVbeta (Clontech) with primer 99.120.1 and 99.120.2 and cloned into PinAI site of CP627.

CP657 is a plasmid with 2.2kb 5' flanking region of human UP II gene in pGL3-Basic (Promega). The 2.2kb hUPII was amplified by PCR from GenomeWalker product with primer 100.113.1 and 100.113.2 and TA-cloned into
25 pGEM-T to generate CP655.

The 2.2kb insert digested from SacII (blunt-ended) and KpnI was cloned into pGL3-Basic at HindIII (blunted) and KpnI to create CP657.

CP1089

5 The 1kb (-965 to +1) mouse UPII was digested by PinAI from CP263 and inserted into CN422 (PSE driving E1A and GKE driving E1B with the deletions of E1A/E1B endogenous promoter) cut by PinAI and purified and further digested with EagI and ligated with 1kb (-1128 to +1) human UPII cut from CP669 with EagI.

CP1129

10 The 1.8kb hUPII fragment with PinAI site was amplified from CP657 with primer 127.50.1 and 127.2.2 and cloned into PinAI site of CP629.

CP1131

15 CP686 was constructed by replacing the CMV promoter in CP629 with an AFP fragment from CP219. A 1.4kb DNA fragment was released from CP686 by digesting it with BssHII, filling with Klenow, then digesting with BglII. This DNA fragment was then cloned into a similarly cut CP686 to generate CP1199. In CP1199, most of the E1B 19-KDa region was deleted. The 1.8kb hUPII fragment with PinAI site was amplified from CP657 by PCR with primer 127.50.1 and 127.2.2 and inserted into similarly digested CP1199 to create CP1131.

20 The plasmids above were all co-transfected with pBHGE3 to generate CV874 (from CP1086), CV875 (from CP1087), CV876 (from 1088) and CV877 (from CP1089), CV882 (from CP1129) and CV884 (from CP1131). CP1088, CP1129 and CP1131 were cotransfected with pBHGE3 for construction of CV876, CV892 and CV884, respectively by lipofectAMINE (Gibco/BRL) for 11-14 days.

25 pBHGE3 was purchased from Microbix, Inc., and was described previously. The cells were lysed by three freeze-thaw cycles and plaqued on 293 cells for a week.

The single plaques were picked and amplified by infection in 293 cells for 3-5 days. The viral DNAs were isolated from the lysates and the constructs were confirmed by PCR with primer 31.166.1/ 51.176 for CV876 and primer 127.50.1/51.176 for CV882 and CV884 at E1 region and primer 32.32.1/2 for all three viruses at E3 region.

TABLE 5

Name	Vector	Ad 5 Vector	E1A TRE	E1B TRE	E3
CV874	CP1086	pBHGE3	1.9 kb mUPII	IRES	intact
CV875	CP1087	pBHGE3	1.0 kb hUPII	IRES	intact
CV876	CP1088	pBHGE3	2.2 kb hUPII	IRES	intact
CV877	CP1089	pBHGE3	1.0 kb mUPII	1.0 kb hUPII (E1B promoter deleted)	intact
CV882	CP1129	pBHGE3	1.8 kb hUPII	IRES	intact
CV884	CP1131	pBHGE3	1.8 kb hUPII	IRES (E1B 19-kDa deleted)	intact

Viruses are tested and characterized as described above.

Primer sequences:

96.74.1	GACGTCGACATCGTGTTTTTCAAAGGAA
96.74.2	GACGTCGACTAATTCCGGTTATTTTCCA
96.74.3	CCTGAGACGCCCGACATCACCTGTG
96.74.4	TGCTGAATGGTCGACATGGAGGCTTGGGAG
96.74.5	CACAACCGCTCTCCACAGATGCATG
96.74.6	GTCGACCATTTCAGCAAACAAAGGCGTTAAC
100.113.1	AGGGGTACCCACTATAGGGCACGCGTGGT
100.113.2	ACCCAAGCTTGGGATGCTGGGCTGGGAGGTGG
127.2.2	AGGACCGGTGGGATGCTGGGCTGGGAGGTGG
127.50.1	AGGACCGGTCAGGCTTACCCCAGACCCAC
31.166.1	TGCGCCGGTGTACACAGGAAGTGA
32.32.1	GAGTTTGTGCCATCGGTCTAC

32.32.2	AATCAATCCTTAGTCCTCCTG
51.176	GCAGAAAAATCTTCCAAACACTCCC
99.120.1	ACGTACACCGGTCGTTACATAACTTAC
99.120.2	CTAGCAACCGGTCGGTTCATAAACG

Example 5: *In vitro* and *In vivo* assays of anti-tumor activity

An especially useful objective in the development of urothelial cell-specific adenoviral vectors is to treat patients with bladder cancer. An initial indicator of the feasibility is to test the vector(s) for cytotoxic activity against cell lines and tumor xenografts grown subcutaneously in Balb/c nu/nu mice.

***In vitro* characterization of CV876**

Virus yield assay for CV876

5 X 10⁵ 293, RT-4, SW780, PA-1, G361, MKN1, HBL-100, Fibroblast (from lung) and Smooth muscle cells (from bladder) were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Unlike wt. Ad5, CV802 which grows well in all of the cells tested, CV876 replicates much better in permissive cells (293, RT-4 and SW780) than in non-permissive cells (PA-1, G361, MKN1, HBL-100 and primary cells) by about 100-10000 fold. Noticeably, the replication in SW780 for CV876 is about 100 fold less than CV802, which indicates the limitation of this virus in efficacy.

Growth curve experiment for CV876

5 X 10⁵ RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with 133) or CV876 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV876 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. However, CV876 does show a delay of replication in RT-4 compared to CV802.

Cytopathic effect assay for CV876

5 X 10⁵ 293, RT-4, SW780, PA-1, MKN1 and LNCap were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV876 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV876 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (PA-1, MKN-1 and LNCap).

MTT assay for CV876

2 X 10⁴293, RT-4, SW780, MKN1, PA-1, HBL-100, Smooth muscle cells (from bladder) and Fibroblast (from lung) were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV876 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time point of day 1, 3,5,7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV876 shows efficacy only in permissive cells but not in non-permissive cells. Again, in RT-4 and SW780, CV876 kills the cells much slower than CV802.

In vitro characterization of CV882

Virus yield assay for CV882

5 X 10⁵293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV882 in permissive cells (293, RT-4 and SW780) is comparable to CV802 (the difference is less than 100 fold) while it shows over 1000-

1000000 fold difference in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells).

Growth curve experiment for CV882

5 X 10⁵ RT-4, PA-1, and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at a MOI of 2 pfu/cell. After a 4h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV882 replicates well only in RT-4 but not in primary cells and PA-1 over a 120h period of time. Additionally, CV882 shows better replication in RT-4 compared to CV876.

Cytopathic effect assay for CV882

5 X 10⁵ 293, RT-4, SW780, HBL-100, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV882 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV882 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (HBL-100, G361, PA-1 and Fibroblast cells).

MTT assay for CV882

2 X 10⁴ RT-4, SW780, PA-1, HBL-100, U118 and Fibroblast were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV882 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution, which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV882 shows efficacy only in permissive cells but not in non-permissive cells.

In Vitro Characterization of CV884

Virus yield assay for CV884

5 X 10⁵ 293, RT-4, SW780, G361, LNCap, HBL-100, MKN1, PA-1, Fibroblast and Smooth muscle cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV984 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. After an additional 72h at 37°C, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

The replication of CV884 is very similar as CV802 in permissive cells (293, RT-4 and SW780) but shows over 1000 fold difference with CV802 in non-permissive cells (G361, LNCap, HBL-100, MKN1, PA-1 and primary cells). CV884 shows better efficacy than CV876 and CV882 without losing much specificity.

Growth curve experiment for CV884

5 X 10⁵ RT-4, PA-1, Smooth muscle and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at a MOI of 2 pfu/cell. After a 4-h incubation at 37°C, cells were washed with prewarmed PBS, and 2ml of complete RPMI 1640 were added to each well. At different time points of 0, 12, 24, 36, 48, 72, 96 and 120h, the cells were scraped into medium and lysed by three freeze-thaw cycles. The lysates were tested for virus production by triplicate plaque assay for 8-10 days under semisolid agarose on 293 cells.

Very similar as in virus yield assay, CV884 replicates very well only in RT-4 (similar as CV802) but not in primary cells and PA-1. Again, the replication of CV884 is better than CV882 and CV876.

Cytopathic effect assay for CV884

5 X 10⁵ 293, RT-4, SW780, G361, PA-1 and Fibroblast cells were plated into each well of six-well plates. Twenty-four hours later, medium was aspirated and replaced with 1ml of serum-free RPMI 1640 containing CV802 (wt.Ad5 with E3) or CV884 at increasing MOI from 0.001 to 10 (the data shown was at MOI 1). After a 4-h incubation at 37°C, medium was replaced with 3ml of complete RPMI 1640 and incubated at 37°C for 6-8 days when cytopathic effect was observed for CV802 at MOI 0.01.

CV802 shows efficacy in all the cells tested while CV884 only kills the permissive cells (293, RT-4 and SW780) but not the non-permissive cells (G361, PA-1 and Fibroblast cells).

MTT assay for CV884

2 X 10⁴293, RT-4, SW780, U118, Fibroblast and Smooth muscle cells were plated into each well of 96-well plates. Twenty-four hours later, the cells were infected with CV802 and CV884 at increasing MOI from 0.001 to 10 in complete RPMI 1640. A rapid colorimetric assay for cell growth and survival was run at different time points of day 1, 3, 5, 7 and 10. The medium was replaced by 50ul of MTT at 1mg/ml solution which is converted to an insoluble purple formazan by dehydrogenase enzymes present in active mitochondria of live cells. After 3-4h incubation at 37°C, the solution was replaced by isopropanol and the plates were incubated at 30°C for 1h and read at 560nm test wavelength and 690nm reference wavelength.

Similar as the results in CPE assay, CV884 shows strong efficacy (similar as wt. Ad5) only in permissive cells but not in non-permissive cells.

In vivo activity of CV808

Mice were given subcutaneous (SC) injections of 1 x 10⁶ sW780 cells. When tumors grew to about 500 mm³, CV808 was introduced into the mice (5 X 10⁷ PFU of virus in 0.1 ml PBS and 10% glycerol) intratumorally. Control mice received vehicle alone. Tumor sizes were measured weekly. The results are shown in FIG. 11. The data indicate that CV808 was effective at suppressing tumor growth.

While it is highly possible that a therapeutic based on the viruses described here would be given intralesionally (i.e., direct injection), it would also be desirable to determine if intravenous (IV) administration of adenovirus vector can affect tumor growth. If so, then it is conceivable that the virus could be used to treat metastatic tumor deposits inaccessible to direct injection. For this experiment, groups of mice bearing bladder epithelial tumors are inoculated with 10⁸ to 10¹⁰ PFU of an adenoviral vector by tail vein injection, or with buffer used to carry the virus as a negative control. The effect of IV injection of the adenoviral vector on tumor size is compared to vehicle treatment.

Example 6: Adenoviral vectors with adenovirus death protein (ADP) under the control of a urothelial-cell specific TRE

An adenovirus in which the ADP gene is under control of a urothelial cell-specific TRE can be constructed as described below. ADP is encoded within the E3 region and naturally under control of the major late promoter (MLP). The gene appears to code for a protein (ADP) that is important in expediting host cell lysis. Tollefson et al. (1996) *J. Virol.* 70(4):2296; Tollefson et al. (1992) *J. Virol.* 66(6):3633. Thus, adenoviral vectors containing the ADP gene may render the adenoviral vector more potent, making possible more effective treatment and/or a lower dosage requirement.

The ADP coding sequence from Ad2 can be introduced into Ad5 in the E3 region (which is often deleted in the constructs; see Example 1), as follows.

An ADP cassette is constructed using overlap PCR. The Y leader, an important sequence for correct expression of some late genes, is PCR amplified using primers:

5' GCCTTAATTAAAAGCAAACCTCACCTCCG...Ad2 28287bp
(37.124.1) (SEQ ID NO: ____); and

5' GTGGAACAAAAGGTGATTAAAAAATCCCAG...Ad2 28622bp
(37.146.1) (SEQ ID NO: ____).

The ADP coding region is PCR amplified using primers

5' CACCTTTTGTTCACCGCTCTGCTTATTAC...Ad2 29195bp
(37.124.3) (SEQ ID NO: ____)

5' GGCTTAATTAAGTGTGAAAGGTGGGAGC...Ad2 29872bp (37.124.4)
(SEQ ID NO: ____).

The two fragments were annealed and the overlap product was PCR amplified using primers 37.124.1 and 37.124.4. The ends of the product were polished with Klenow fragment and ligated to BamHI cut pGEM-72(+) (Promega, Madison, WI) to produce CN241. The ADP cassette was excised by digesting CN241 with PacI

restriction endonuclease and ligated with two vectors, CN247 and CN248, generating plasmids CN252 and CN270, respectively.

CN247 contains a unique PacI site in the E3 region and was constructed as follows. A plasmid containing the full length Ad5 genome, TG3602 (Transgene, France), was digested with BamHI and relegated to yield CN221. The backbone of this plasmid (outside of the Ad5 sequence) contained a PacI site that needed to be removed to enable further manipulations. This was effected by digesting CN221 with PacI and polishing the ends with T4 DNA polymerase, resulting in CN246. CN246 was digested with AscI and AvrII (to remove intact E3 region). This fragment was replaced by a similarly cut fragment derived from BHG11. The resulting plasmid, CN247, lacks the E3 region and has a PacI site suitable for insertion of the ADP cassette fragment (described above). Ligation of CN247 with the ADP cassette generated CN252.

CN248 (a construct that would allow introduction of an ADP cassette into a Ad that also contains a deletion/substitution in the E4 region) was made as follows. The E4 region was deleted by digesting CN108, a construct that contains right hand end Ad5 sequence from the unique EcoRI site in the E3 region, with AvrII and AflII. The only E4 ORF necessary for viral replication, ORF 6, was reintroduced by PCR amplifying the ORF with primers,

33.81.1 (Ad5 33096):

GCAGCTCACTTAAGTTCATGTCG (SEQ ID NO:_)

33.81.2 (Ad5 34084):

TCAGCCTAGGAAATATGACTACGTCCG (SEQ ID NO:_)

The resulting plasmid is CN203. CN203 was digested with EcoRI and ligated to CN209, a shuttle plasmid, to generate CN208. In the final cloning step, CN208 was digested with AscI and AvrII and ligated to similarly cut E4 deletion/substitution with the ADP cassette.

Thus, both CN252 and CN270 are adenoviral derivatives containing the ADP and lacking the E3 gene. In addition, CN270 lacks some sequence in the E4 region as previously described. Full-length adenoviral vectors are obtained via *in vitro* ligation of (1) appropriately prepared viral DNA digested with BamHI and (2) CN252 or
5 CN257 also digested with BamHI. The ligation product is used to transfect 293 cells. Plaque assays are performed as described above.

CN252 and CN270 can also be modified by insertion of a UP-TRE fragment to place the ADP gene under control of UP-TRE.

Although the foregoing invention has been described in some detail by way of
10 illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications can be practiced. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.

TABLE 6 IRES SEQUENCES

A 519 base pair IRES obtainable from encephelomyocarditis virus

(EMCV) SEQ ID NO: __.

5 1 GACGTCGACTAATTCCGGTTATTTCCACCATATTGCCGTCTTTTGGCAA
Sall
51 TGTGAGGGCCCGAAACCTGGCCCTGTCTTCTTGACGAGCATTCTAGGG
101 GTCTTTCCCCTCTCGCCAAAGGAATGCAAGGTCTGTTGAATGTCGTGAAG
151 GAAGCAGTTCTCTGGAAGCTTCTTGAAGACAAACAACGTCTGTAGCGAC
201 CCTTTCAGGCAGCGGAACCCCCACCTGGCGACAGGTGCCTCTGCGGCC
10 251 AAAAGCCACGTGTATAAGATACACCTGCAAAGGCGGCACAACCCAGTGC
301 CACGTTGTGAGTTGGATAGTTGTGGAAAGAGTCAAATGGCTCTCCTCAAG
351 CGTATTCAACAAGGGGCTGAAGGATGCCCAGAAGGTACCCATTGTATGG
401 GATCTGATCTGGGGCCTCGGTGCACATGCTTTACATGTGTTTAGTCGAGG
451 TTAAAAAACGTCTAGGCCCCCGAACCACGGGGACGTGGTTTTTCCTTTGA
15 Sall
501 AAAACACGATGTCGACGTC

An IRES obtainable from vascular endothelial growth factor (VEGF) SEQ

ID NO: __.

20 1 ACGTAGTCGACAGCGCAGAGGCTTGGGGCAGCCGAGCGGCAGCCAGGCCC
Sall
51 CGGCCCCGGGCCTCGGTTCCAGAAGGGAGAGGAGCCCGCCAAGGCGCGCAA
101 GAGAGCGGGCTGCCTCGCAGTCCGAGCCGGAGAGGGAGCGCGAGCCGCGC
25 151 CGGCCCCGACGGCCTCCGAAACCATGGTTCGACACGTA
Sall

A 5'UTR region of HCV SEQ ID NO:___.

1
GCCAGCCCCCTGATGGGGGCGACACTCCGCCATGAATCACTCCCCTGTGAGGAACTACTG
61
5 TCTTCACGCAGAAAGCGTCTAGCCATGGCGTTAGTATGAGTGTCTGTCAGCCTCCAGGAC
121 CCCCCCTCCCGGGAGAGCCATAGTGGTCTGCGGAACCGGTGAGTACACCGGAATTGCCAG
181 GACGACCGGGTCCTTTCTTGGATTAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCC
241 GCAAGACTGCTAGCCGAGTAGTGTGGGTGCGGAAAGGCCTTGTGGTACTGCCTGATAGG
301 GTGCTTGCAGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACC (341)

A 5'UTR region of BiP SEQ ID NO:___

1
CCCGGGGTCACTCCTGCTGGACCTACTCCGACCCCCCTAGGCCGGGAGTGAAGGCGGGACT
61
15 TGTGCGGTTACCAGCGGAAATGCCTCGGGGTCAGAAGTCGCAGGAGAGATAGACAGCTGC
121 TGAACCAATGGGACCAGCGGATGGGGCGGATGTTATCTACCATTGGTGAACGTTAGAAAC
181 GAATAGCAGCCAATGAATCAGCTGGGGGGCGGAGCAGTGACGTTTATTGCGGAGGGGGC
241 CGCTTCGAATCGGCGGGCGGCCAGCTTGGTGGCCTGGGCCAATGAACGGCCTCCAACGAGC
301 AGGGCCTTCACCAATCGGCGGCCCTCCACGACGGGGCTGGGGGAGGGTATATAAGCCGAGT
20 361 AGGCGACGGTGAGGTCGACGCCGGCCAAGACAGCACAGACAGATTGACCTATTGGGGTGT
421 TTCGCGAGTGTGAGAGGGAAGCGCCGCGGCCTGTATTTCTAGACCTGCCCTTCGCCTGGT
481 TCGTGGCGCCTTGTGACCCCGGGCCCCCTGCCGCCTGCAAGTCGAAATTGCGCTGTGCTCC
541 TGTGCTACGGCCTGTGGCTGGACTGCCTGCTGCTGCCCCAACTGGCTGGCAAGATG (595)

A 5'UTR of PDGF SEQ ID NO:___.

1
GTTTGCACCTCTCCCTGCCCGGGTGCTCGAGCTGCCGTTGCAAAGCCAACTTTGAAAAA

61
GTTTTTTTGGGGGAGACTTGGGCCTTGAGGTGCCCAGCTCCGCGCTTTCCGATTTTGGGGG

5
121 CTTTCCAGAAAATGTTGAAAAAAGCTAAGCCGGCGGGCAGAGGAAAACGCCTGTAGCCG

181 GCGAGTGAAGACGAACCATCGACTGCCGTGTTTCCTTTTCTCTTGGAGGTTGGAGTCCCC

241 TGGGCGCCCCCACACCCCTAGACGCCTCGGCTGGTTCGCGACGCAGCCCCCGGCCGTGG

301 ATGCTGCACTCGGGCTCGGGATCCGCCCAGGTAGCCGGCCTCGGACCCAGGTCTGCGCC

10
361 CAGGTCCTCCCCCTGCCCCCAGCGACGGAGCCGGGGCCGGGGCGGGCGGCCGGGGGCA

421 TCGGGGTGAGCCGCGGCTGCAGAGGCCTGAGCGCCTGATCGCCGCGGACCTGAGCCGAGC

481 CCACCCCCCTCCCCAGCCCCCACCCTGGCCGCGGGGGCGGGCGGCTCGATCTACGCGTC

541 CGGGGCCCCGCGGGGCCGGGCCCGGAGTCGGCATG (575)

TABLE 7 LITERATURE REFERENCES FOR IRES

IRES Host	Example	Reference
Picornavirus	HAV	Glass et al., 1993. Virol 193:842-852
	EMCV	Jang & Wimmer, 1990. Gene Dev 4:1560-1572
	Poliovirus	Borman et al., 1994. EMBO J 13:3149-3157
HCV and pestivirus	HCV	Tsukiyama-Kohara et al., 1992. J Virol 66:1476-1483
	BVDV	Frolov I et al., 1998. RNA. 4:1418-1435
Leishmania virus	LRV-1	Maga et al., 1995. Mol Cell Biol 15:4884-4889
Retroviruses	MoMLV VL30 (Harvey murine sarcoma virus)	Torrent et al., 1996. Hum Gene Ther 7:603-612
	REV	Lopez-Lastra et al., 1997. Hum Gene Ther 8:1855-1865
Eukaryotic mRNA	BiP	Macejak & Sarnow, 1991. Nature 353:90-94
	antennapedia mRNA	Oh et al., 1992. Gene & Dev 6:1643-1653
	FGF-2	Vagner et al., 1995. Mol Cell Biol 15:35-44
	PDGF-B	Bernstein et al., 1997. J Biol Chem 272:9356-9362
	IGFII	Teerink et al., 1995. Biochim Biophys Acta 1264:403-408
	eIF4G	Gan & Rhoads, 1996. J Biol Chem 271:623-626
	VEGF	Stein et al., 1998. Mol Cell Biol 18:3112-3119; Huez et al., 1998. Mol Cell Biol 18:6178-6190